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(54) Title: THERMOSTABLE DNA POLYMERASE FROM THERMOANAEROBACTER THERMOHYDROSULFURICUS

(57) Abstract

An enzymatically active DNA polymerase or fragment thereof having at least 80 % homology in its amino acid sequence to at least a contiguous 40 amino acid sequence of the DNA polymerase of *Thermoanaerobacter thermohydrosulfuricus*.

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DESCRIPTION

THERMOSTABLE DNA POLYMERASE FROM
THERMOANAEROBACTER THERMOHYDROSULFURICUS

5   This application claims priority to Mamone et al., U.S.
Serial   No. 08/766,014 filed December 13, 1996, entitled
"Thermostable   DNA   Polymerase   From   Thermoanaerobacter
Thermohydrosulfuricus" which claims priority to U.S. Serial
No.   60/008,688,   filed   December   15,   1995,   entitled
10   "Thermostable DNA Polymerase."   These references are herein
incorporated by reference in their entirety including any
figures, tables, or drawings.

Background of the Invention
          The present invention relates to novel thermostable DNA
15   polymerases   obtainable   from   the   thermophilic   anaerobe
Thermoanaerobacter   thermohydrosulfuricus,   to   certain
deletants and mutants of this enzyme, to genes and vectors
encoding the wild type and mutant polymerases and their use
in strand displacement activity, polymerase chain reaction,
20   DNA sequencing and as reverse transcriptases.
          The following is a discussion of the relevant art, none
of which is admitted to be prior art to the appended claims.
          DNA polymerases are a family of enzymes involved in DNA
repair and replication.   DNA polymerases have been isolated
25   from E.coli (e.g. E.coli DNA polymerase I and the Klenow
fragment thereof) and T4 DNA polymerase and more recently
thermostable DNA polymerases have been isolated (e.g. from
T. aquaticus, US Patent 4,889,818, and from T. litoralis).
Thermostable DNA polymerases have been suggested (US Patent
30   4,683,195)   for   use   in   amplifying   existing   nucleic   acid
sequences   in   amounts   that   are   large   compared   to   that
originally present.   The polymerase chain reaction (PCR) and
strand displacement amplification (SDA) are two methods of
amplifying nucleic acid sequences.

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PCR is based on the hybridization of oligonucleotide primers to specific sequences on opposite strands of the target DNA molecule, and subsequent extension of these primers with a DNA polymerase to generate two new strands of

5   DNA which themselves can serve as a template for a further round of hybridization and extension. In PCR reactions the product of one cycle serves as the template for the next cycle such that at each repeat of the cycle the amount of the specific sequence present in the reaction doubles

10   leading to an exponential amplification process.

PCR relies on a process of temperature cycling to promote the amplification reaction. This temperature cycling is necessary to separate the strands of DNA formed in one cycle of the reaction to allow hybridization of the

15   oligonucleotides required to initiate the next cycle. DNA strand separation is usually achieved by melting the DNA at temperatures in the range 90-100°C followed by cooling to a lower temperature to allow oligonucleotide hybridization followed by extension or ligation, depending on the reaction

20   process. This cycling process may be repeated 20-50 times, again depending on the process and the degree of amplification required.

Temperature cycling is normally achieved by the use of specialized equipment, commonly termed thermocyclers, which

25   can operate by a wide variety of mechanical, electrical or hydraulic means, but serve a common purpose in heating and cooling a small container or a number of such containers in which the amplification reaction is performed.

In order for the amplification reactions to proceed

30   with the desired efficiency and specificity it is necessary to perform the temperature cycling process within strictly defined and reproducible limits of temperature and time. Failure of the temperature cycling apparatus to achieve the required conditions will result in the partial or total

35   failure of the amplification reaction. These strict requirements on time and temperature of cycling can impose

severe restrictions if the handling of large numbers of
reactions is required. If it is desired to perform several
hundred or more reactions simultaneously use of conventional
thermocyclers would be extremely expensive in terms of the
5   capital investment required in equipment, and would in any
case be prone to variations between individual thermo-
cyclers. Alternative solutions for large scale use have been
constructed based on fan assisted oven or multiple water
baths, but such solutions are inevitably cumbersome in use
10   and in any case still require the user to take the normal
stringent steps to avoid evaporation which become extremely
difficult to apply to a large number of cycles.

In reverse transcription/ polymerase chain reaction
(RT/PCR), a DNA primer is hybridized to a strand of the
15   target RNA molecule, and subsequent extension of this primer
with a reverse transcriptase generates a new strand of DNA
which can serve as a template for PCR. Preparation of the
DNA template is preferably carried out at an elevated
temperature to avoid early termination of the reverse
20   transcriptase reaction caused by RNA secondary structure.
There is a lack of efficient reverse transcriptases that act
at elevated temperatures, e.g. above 50 °C.

SDA   differs   from   PCR   in   being   an   isothermal
amplification process, i.e. all reactions occur at the same
25   temperature without the need for elevated temperature to
melt DNA strands. This is made possible by adoption of a
reaction scheme which uses the ability of certain DNA
polymerases when extending along a DNA template strand to
displace any DNA molecules already hybridized to the
30   template.   In SDA this strand displacement is used to
separate the double stranded DNA produced during the
reaction process and hence to maintain continuous
amplification of the target DNA sequence (Walker, G.T.,
Little, M.C., Nadeau, J.G. and Shank D.D. (1992) *Proc. Natl.*
35   *Acad. Sci. USA* 89:392-396). SDA is therefore in principle
more suited to use with large numbers of samples than PCR as

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the isothermal process, which is performed at temperatures
of 37 °C to 60 °C, does not require stringent precautions to
be taken to avoid evaporation and can be performed with
simple temperature control equipment, for example in a
5   standard laboratory incubator.

DNA polymerases, e.g. Sequenase, Klenow, Taq, etc, have
also been extensively used in DNA sequencing, see for
example "Molecular Cloning: A Laboratory Manual" (Sambrook,
Fritsch, and Maniatis, 2nd edition, Cold Spring Harbor
10   laboratory Press, 1989).

Summary of the Invention

The present invention provides a thermostable DNA
polymerase from Thermoanaerobacter thermohydrosulfuricus.
This enzyme is useful for procedures requiring
15   strand-displacing DNA synthesis such as SDA, for DNA
sequencing, reverse transcription and polymerase chain
reaction. Included within the scope of the present
invention are various mutants (deletion and substitution)
that retain thermostability and the ability to replicate DNA
20   with substantially the same efficiency as the native
Thermoanaerobacter thermohydrosulfuricus polymerase.

In a first aspect, the present invention provides a
purified DNA polymerase or fragment thereof having the DNA
polymerase activity of Thermoanaerobacter thermo-
25   hydrosulfuricus and having at least 80% amino acid homology,
preferably at least 90% homology, to at least a contiguous
40 amino acid sequence shown in Fig. 2 A-D (SEQ. ID. NO.
2). Fig. 2A-D represents the translation of an open reading
frame spanning nucleotides 1056-3674 of genomic DNA
30   encoding a thermostable DNA polymerase from
Thermoanaerobacter thermohydrosulfuricus (Fig. 1A-E)(SEQ.
ID. NO. 1) potentially encoding the native polymerase.

When used herein, the term amino acid homology means
the amino acid identity of the parent enzyme or conservative
35   amino acid changes thereto. The DNA polymerase can be

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encoded by a full-length nucleic acid sequence or any
portion of the full-length nucleic acid sequence, so long as
a functional activity of the polypeptide is retained. The
amino acid sequence will be substantially similar to the
5 sequence shown in Fig. 2A-D, or fragments thereof. A
sequence that is substantially similar will preferably have
at least 80% identity (more preferably at least 90% and most
preferably 98-100%) to the sequence of Fig. 2A-D.

By "identity" is meant a property of sequences that
10 measures their similarity or relationship. Identity is
measured by dividing the number of identical residues by the
total number of residues and multiplying the product by 100.
Thus, two copies of exactly the same sequence have 100%
identity, but sequences that are less highly conserved, and
15 have deletions, additions, or replacements, may have a lower
degree of identity. Those skilled in the art will recognize
that several computer programs are available for determining
sequence identity.

The purified enzyme of the present invention has a
20 molecular weight of approximately 90,000 daltons when
measured on SDS-PAGE. It possesses a 5'-3' exonuclease
activity. The temperature optimum of DNA synthesis is near
75 °C under assay conditions. The optimum magnesium ion and
manganese ion concentrations for DNA synthesis are 1 mM and
25 0.5 mM respectively.

The term thermostable polymerase means an enzyme which
is stable to heat (and heat resistant) and is suitable for
use in SDA and/or sequencing at an elevated temperature, for
example 70 °C. The thermostable enzyme herein must satisfy a
30 single criterion to be effective for the amplification
reaction, i.e., the enzyme must not become irreversibly
denatured (inactivated) when subjected to the elevated
temperatures for the time necessary to effect amplification.
Irreversible denaturation for purposes herein refers to
35 permanent and complete loss of enzymatic activity.
Preferably, the enzyme will not become irreversibly

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denatured at about 70 °C but may become denatured at higher
temperatures. The thermostable enzyme herein preferably has
an optimum temperature at which it functions that is higher
than about 40 °C, which is the temperature below which
5   hybridization of primer to template is promoted, although,
depending on (1) salt concentration and composition and (2)
composition and length of primer, hybridization can occur at
higher temperature (e.g., 45-70 °C). The higher the
temperature optimum for the enzyme, the greater the
10 specificity and/or selectivity of the primer-directed
extension process. However, enzymes that are active below
40 °C, e.g., at 37 °C, are also within the scope of this
invention provided they are heat stable. Preferably, the
optimum temperature ranges from about 50 to 80 °C, more
15 preferably 50-70 °C.

        When used herein, the term a DNA polymerase or fragment
thereof having the DNA polymerase activity of
Thermoanaerobacter thermohydrosulfuricus means a DNA
polymerase or fragment thereof (as hereinafter defined)
20 which has the ability to replicate DNA with substantially
the same efficiency as the enzyme encoded by the SEQ. ID.
NO. 1. By substantially the same efficiency is meant at
least 80% and preferably at least 90% of the efficiency of
the enzyme encoded by SEQ. ID. NO. 1 to incorporate deoxy-
25 nucleotides.

        The invention also encompasses a stable enzyme
composition which comprises a purified thermostable DNA
polymerase from *Thermoanaerobacter thermohydrosulfuricus* in
a buffer.

30   The DNA polymerases of the present invention are
preferably in a purified form. By purified is meant that
the DNA polymerase is isolated from a majority of host cell
proteins normally associated with it; preferably the
polymerase is at least 10% (w/w), e.g. at least 50% (w/w),
35 of the protein of a preparation, even more preferably it is
provided as a homogeneous preparation, e.g.. homogeneous

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solution.   Preferably the DNA polymerase is a single
polypeptide on an SDS polyacrylamide gel.

5   Buffers around neutral pH (5-9) such as 5-100 mM Tris-
HCl, phosphate or MES are suitable for use in the current
invention.

The present invention also provides a gene encoding a
polymerase of the present invention.   Fig. 1A-E represents
nucleotides 1-5300 of a cloned genomic sequence encompassing
the gene encoding the polymerase of the present invention
10   (SEQ. ID. NO. 1).   Fig. 7A-C represents the DNA sequence of
a full length native polymerase from *Thermoanaerobacter
thermohydrosulfuricus* (SEQ. ID. NO. 23).

It has been found that the entire amino acid sequence
of the polymerase is not required for enzymatic activity.
15   Thus, for example, the exonuclease domain of the enzyme has
been deleted to give an enzyme of molecular weight of
approximately 64,000 daltons when measured on SDS-PAGE which
retains enzyme activity and also has reverse transcriptase
activity making it useful for making cDNA.   This
20   exonuclease-free enzyme is analogous to the Klenow fragment
of *E. coli* DNA polymerase I.   Thus, the present invention
also provides fragments of the polymerase which retain the
DNA   polymerase   activity   of   *Thermoanaerobacter
thermohydrosulfuricus* but have one or more amino acids
25   deleted, preferably from the amino-terminus, while still
having at least 80% amino acid homology to at least a 40
contiguous amino acid sequence shown in Fig. 2A-D (SEQ. ID.
NO. 2).

In a further aspect, the present invention provides a
30   thermostable DNA polymerase which corresponds to the DNA
polymerase from *Thermoanaerobacter thermohydrosulfuricus* in
which up to one third of the amino acid sequence at the
amino-terminus has been deleted.   In particular, fragments
of   *Thermoanaerobacter   thermohydrosulfuricus*   having
35   N-terminal deletions to give 578 amino acids (See Fig. 4A-C)

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(SEQ. ID. NO. 4) and 608 amino acids (See Fig. 3) (SEQ. ID. NO. 3) have been found to retain enzyme activity.

It is preferred that the 5'-3' exonuclease activity of the DNA polymerase is removed or reduced. This may be achieved by deleting the amino acid region of the enzyme responsible for this activity, e.g. by deleting up to one third of the amino acid sequence at the amino terminus, or by appropriate amino acid changes.

In addition to the N-terminal deletions and amino acid changes to remove the exonuclease activity, the enzyme may have conservative amino acid changes compared with the native enzyme which do not significantly influence thermostability or enzyme activity. Such changes include substitution of like charged amino acids for one another or amino acids with small side chains for other small side chains, e.g. ala for val. More drastic changes may be introduced at non-critical regions where little or no effect on polymerase activity is observed by such a change.

Joyce and Steitz, Annu. Rev. Biochem, 63:777-822, 1994, discuss various functions of DNA polymerases including the catalytic center, the binding site for the 3' terminus of the primer, and the dNTP binding site. In particular, it mentions mutations that affect the binding of dNTP in the ternary complex. European Patent Application 0655506A discloses that the presence of a polar, hydroxyl containing amino acid residue at a position near the binding site for the dNTP substrate is important for the polymerase being able to efficiently incorporate a dideoxynucleotide. Applicant has discovered that the modification of the dNTP binding site for the dNTP substrate in DNA polymerase obtainable from *Thermoanaerobacter thermohydrosulfuricus* by the inclusion of a polar, hydroxyl containing amino acid residue at a position near the binding site increases the efficiency of the polymerase to incorporate a dideoxynucleotide. Preferably the polar, hydroxyl containing amino acid is tyrosine. It has also been found

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that replacing the phenylalanine at the position corresponding to 706 of the native enzyme with tyrosine improves the incorporation of dideoxynucleotides when the enzyme is used for sequencing. In particular, a polymerase
5   from *Thermoanaerobacter thermohydrosulfuricus* in which the exonuclease activity has been deleted e.g. by point mutation or deletion and which has the phenylalanine at the position corresponding to 706 of the native enzyme replaced by an amino acid which increases the efficiency of the enzyme to
10   incorporate dideoxynucleotides at least 20 fold compared to the wild type enzyme, e.g. tyrosine, is a particularly preferred enzyme for use in sequencing. Preferably, this modified enzyme has from between 540 and 582 amino acids, for example 560 to 582 amino acids and preferably 578 amino
15   acids.

The DNA polymerases of the present invention can be constructed using standard techniques familiar to those who practice the art. By way of example, in order to prepare a polymerase with the phenylalanine to tyrosine mutation,
20   mutagenic PCR primers can be designed to incorporate the desired Phe to Tyr amino acid change (FY mutation in one primer). Deletion of the exonuclease function is carried out by PCR to remove the amino terminus, or standard techniques of site directed mutagenesis to generate point
25   mutations.

Improved expression of the DNA polymerases of the present invention can be achieved by introducing silent codon changes (i.e., the amino acid encoded is not changed). Such changes can be introduced by the use of mutagenic PCR
30   primers. Silent codon changes such as the following increase protein production in *E. coli*:
substitution of the codon GAG for GAA;
substitution of the codon AGG, AGA, CGG or CGA for CGT or CGC;
35   substitution of the codon CTT, CTC, CTA, TTG or TTA for CTG;
substitution of the codon ATA for ATT or ATC;

substitution of the codon GGG or GGA for GGT or GGC.

Genes encoding the DNA polymerase from *Thermoanaerobacter thermohydrosulfuricus* polymerases in which up to one third of the amino acid sequence at the amino terminus has been deleted and which have the exonuclease activity removed by point mutation and such polymerases which incorporate the phenylalanine to tyrosine modification are also provided by the present invention.

In a yet further aspect, the present invention provides a host cell comprising a vector containing the gene encoding the DNA polymerase activity of the present invention, e.g., encoding an amino acid sequence corresponding to native *Thermoanaerobacter thermohydrosulfuricus* or differentiated from this in that it lacks up to one third of the N-terminal amino acids and optionally has phenylalanine at position 706 replaced by tyrosine.

The DNA polymerases of the present invention are suitably used in SDA, preferably in combination with a thermostable restriction enzyme. Accordingly, the present invention provides a composition which comprises a DNA polymerase of the present invention in combination with a thermostable restriction enzyme, for example *Bso*BI from *Bacillus stearothermophilus*. The invention also features a kit or solution for SDA comprising a DNA polymerase of the present invention in combination and a thermostable restriction enzyme.

The polymerases of the present invention are also useful in methods for generating and amplifying a nucleic acid fragment via a strand displacement amplification (SDA) mechanism. The method generally comprises:

a) specifically hybridizing a first primer 5' to a target nucleic acid sequence, the first primer containing a restriction enzyme recognition sequence 5' to a target binding region;

b) extending the 3' ends of the hybridized material using a DNA polymerase of the present invention, preferably

one in which the exonuclease activity has been removed,
in the presence of three dNTPs and one dNTPα S;

c)   nicking at the hemiphosphorothioate recognition site
       with a restriction enzyme, preferably;

5   d)   extending the 3' end at the nick using a DNA polymerase
       of the present invention, displacing the downstream
       complement of the target strand; and

e)   repeating steps (c) and (d).

10   This SDA method proceeds at a linear amplification
rate if one primer is used as above. However, if two
primers are used which hybridize to each strand of a
double-stranded DNA fragment, then the method proceeds
exponentially (Walker, G.T., Little, M.C., Nadeau, J.G. and
15   Shank D.D. (1992) *Proc. Natl. Acad. Sci. USA* 89:392-396).

       The present invention also provides a method for
determining the nucleotide base sequence of a DNA molecule.
The method includes providing a DNA molecule, annealing with
a primer molecule able to hybridize to the DNA molecule; and
20   incubating the annealed molecules in a vessel containing at
least one, and preferably four deoxynucleotide triphosphate,
and a DNA polymerase of the present invention preferably one
containing the phenylalanine to tyrosine mutation. Also
provided is at least one DNA synthesis terminating agent
25   which terminates DNA synthesis at a specific nucleotide
base. The method further includes separating the DNA
products of the incubating reaction according to size,
whereby at least a part of the nucleotide base sequence of
the DNA molecule can be determined.

30   In preferred embodiments, the sequencing is performed
at a temperature between 40 and 75°C.

       In other preferred embodiments, the DNA polymerase has
less than 1000, 250, 100, 50, 10 or even 2 units of
exonuclease activity per mg of polymerase (measured by
35   standard procedure, see below) and is able to utilize
primers having only 4, 6 or 10 bases; and the concentration

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of all four deoxynucleoside triphosphates at the start of
the incubating step is sufficient to allow DNA synthesis to
continue until terminated by the agent, e.g. a ddNTP.

Preferably, more than 2, 5, 10 or even 100 fold excess
5   of a dNTP is provided to the corresponding ddNTP.

In a related aspect, the invention features a kit or
solution for DNA sequencing including a DNA polymerase of
the present invention and a reagent necessary for the
sequencing such as dITP, deaza dGTP, a chain terminating
10   agent such as a ddNTP, and optionally a pyrophosphatase.

The DNA polymerases of the present invention containing
the phenylalanine to tyrosine mutation are suitably used in
sequencing,   preferably   in   combination   with   a
pyrophosphatase.   Accordingly,   the   present   invention
15   provides a composition which comprises a DNA polymerase of
the present invention containing the phyenylalanine to
tyrosine mutation in combination with a pyrophosphatase,
preferably a thermostable pyrophosphatase from *Thermoplasma
acidophilum*.

20   In another related aspect, the invention features a
method for sequencing a strand of DNA essentially as
described above with one or more (preferably 2, 3 or 4)
deoxyribonucleoside triphosphates, a DNA polymerase of the
present invention, and a first chain terminating agent. The
25   DNA polymerase causes the primer to be elongated to form a
first series of first DNA products differing in the length
of the elongated primer, each first DNA product having a
chain terminating agent at its elongated end, and the number
of molecules of each first DNA products being approximately
30   the same for substantially all DNA products differing in
length by no more than 20 bases. The method also features
providing a second chain terminating agent in the hybridized
mixture at a concentration different from the first chain
terminating   agent,   wherein   the   DNA   polymerase   causes
35   production of a second series of second DNA products
differing in length of the elongated primer, with each

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second DNA product having the second chain terminating agent
at its elongated end. The number of molecules of each second
DNA product is approximately the same for substantially all
second DNA products differing in length from each other by
5   from 1 to 20 bases, and is distinctly different from the
number of molecules of all the first DNA products having a
length differing by no more than 20 bases from that of said
second DNA products.

In preferred embodiments, three or four such chain
10   terminating agents can be used to make different products
and the sequence reaction is provided with a magnesium ion,
or even a manganese or iron ion (e.g. at a concentration
between 0.05 and 100 mM, preferably between 1 and 10mM); and
the DNA products are separated according to molecular weight
15   in four or less lanes of a gel.

In another related aspect, the invention features a
method for sequencing a nucleic acid by combining an
oligonucleotide primer, a nucleic acid to be sequenced,
between one and four deoxyribonucleoside triphosphates, a
20   DNA polymerase of the present invention, and at least two
chain terminating agents in different amounts, under
conditions favoring extension of the oligonucleotide primer
to form nucleic acid fragments complementary to the nucleic
acid to be sequenced.   The method further includes
25   separating the nucleic acid fragments by size and
determining the nucleic acid sequence.   The agents are
differentiated from each other by intensity of a label in
the primer extension products.

In a further aspect, the present invention provides a
30   method for preparing complementary DNA by combining an
oligonucleotide primer, a sample of RNA, a DNA polymerase of
the present invention, and between one and four
deoxyribonucleoside phosphates, under conditions favoring
preparation of the complementary DNA.

35   The DNA polymerases of the present invention which act
as reverse transcriptases lack appreciable, and preferably

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have no RNaseH activity and, as such, are useful in RT/PCR, the generation of hybridization probes and RNA sequencing. In a yet further aspect, the present invention provides a purified thermostable reverse transcriptase having a reverse

5   transcriptase activity of greater than 1000 units per milligram. Preferably, the reverse transcriptase lacks RNaseH activity; the reverse transcriptase is from *Thermoanaerobacter thermohydrosulfuricus*; the reverse transcriptase has an N-terminal deletion or amino acid

10   changes that remove the exonuclease function. In a further aspect, the invention features a method for reverse transcription/polymerase chain reaction (RT/PCR) which utilizes a DNA polymerase of the present invention and a DNA polymerase suitable for PCR in the same reaction vessel.

15   Preferably, the DNA polymerase of the present invention is from *Thermoanaerobacter thermohydrosulfuricus*, the polymerase has one or more amino acids deleted from the amino terminus or amino acid changes to remove the exonuclease activity, the DNA polymerase suitable for PCR is

20   Taq DNA polymerase. In another aspect, the present invention features a kit or solution for RT/PCR comprising a DNA polymerase of the present invention and a DNA polymerase suitable for PCR. Preferably, the DNA polymerase of the present invention is from *Thermoanaerobacter*

25   *thermohydrosulfuricus*, the polymerase has one or more amino acids deleted from the amino terminus or amino acid changes to remove the exonuclease function, and the DNA polymerase suitable for PCR is Taq DNA polymerase.

In a further preferred embodiment the invention

30   features polymerases that have exonuclease activity removed by replacing an aspartic acid residue at position 8 with an alanine residue. This is a preferred mutation for it not only removes the exonuclease activity but removes an RNaseH activity, which may effect reverse transcription.

35   In another aspect, the invention features a method for polymerase chain reaction in the presence of a polymerase

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stabilizing agent, utilizing an enzymatically active DNA polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed.

5   The polymerase stabilizing agents include, but are not limited to, glycerol (10 to 50% final concentration), trimethylamine-N-oxide (TMANO; up to 4 M final concentration), and N-methylmorpholine-N-oxide (MMO; up to 3 M final concentration). Preferably, glycerol is used at a

10   final concentration of 30%. By polymerase stabilizing agent is meant an agent which allows the use of the polymerase in PCR and RT/PCR. These agents reduce the denaturing temperature of the template and stabilize the polymerase. By stabilize is meant make temperature stable. By final

15   concentration is meant the final concentration of the agent in the PCR or RT/PCR solution.

The invention also features kits, with polymerase stabilizing agents, for polymerase chain reaction having an enzymatically active DNA polymerase with at least 80%

20   identity in its amino acid sequence to the DNA polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed. The polymerase stabilizing agents include, but are not limited to, glycerol (10 to 50% final concentration),TMANO (up to 4 M final concentration), and

25   MMO (up to 3 M final concentration). Preferably, glycerol is used at a final concentration of 30%. Also encompassed are solutions for use in polymerase chain reaction, having polymerase stabilizing agents including, but not limited to, glycerol (10 to 50% final concentration), TMANO (up to 4 M

30   final concentration), and MMO (up to 3 M final concentration), and an enzymatically active DNA polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed.

35   Preferably, glycerol is used at a final concentration of 30%.

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In preferred embodiments the exonuclease activity is removed by an N-terminal deletion to give between 540 and 582 amino acids, by deleting up to one third of the amino acid sequence at the N-terminal, by substitutition of an
5   amino acid in the amino terminal one third of the protein, or by substitution of the aspartic acid residue at position 8 with an alanine residue, and the glycerol concentration is 30%.

Applicants have discovered that the DNA polymerases of
10 the present invention can be used to carry out polymerase chain reaction (PCR) when the reaction is carried out in the presence of a polymerase stabilizing agent, such as glycerol, TMANO, or MMO, and the like. Preferably, the glycerol concentration is in the range of 10 to 50%, and
15 most preferably it is at a final concentration of 30%. TMANO and MMO can be used at concentrations up to 4 M and 3 M final concentration, respectively. These agents have been shown to stabilize the polymerase from *Themoanaerobacter thermohydrosulfuricus* (herein and U.S. Patent Application
20 No. 08/766,014, filed December 13, 1996, *supra*). Thus, the polymerases of the present invention are also suitable for reverse transcription/polymerase chain reaction (RT/PCR) under these conditions.

Thus, in another aspect the invention features a method
25 for reverse transcription/polymerase chain reaction, in the presence of a polymerase stabilizing agent, utilizing an enzymatically active DNA polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease
30 activity removed. In preferred embodiments, the polymerase stabilizing agents include, but are not limited to, glycerol (10 to 50% final concentration), TMANO (up to 4 M final concentration), and MMO (up to 3 M final concentration). Preferably, glycerol is used at a final concentration of
35 30%.

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The invention also features kits for reverse transcription/polymerase chain reaction, with a polymerase stabilizing agent, having an enzymatically active DNA polymerase with at least 80% identity in its amino acid
5   sequence to the DNA polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed. Also encompassed are solutions for use in reverse transcription/polymerase chain reaction comprising a polymerase stabilizing agent and an enzymatically active DNA
10   polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed. In preferred embodiments, the polymerase stabilizing agents include, but are not limited to, glycerol (10 to 50% final
15   concentration), TMANO (up to 4 M final concentration), and MMO (up to 3 M final concentration). Preferably, glycerol is used at a final concentration of 30%.

In preferred embodiments the exonuclease activity is removed by having an N-terminal deletion to give between 540
20   to 582 amino acids, by deleting up to one third of the amino acid sequence at the N-terminal, by substitutition of an amino acid in the amino terminal one third of the protein, by substitition of the aspartic acid residue at position 8 with an alanine residue, and the glycerol concentration is
25   30%.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments, and from the claims.

30   Description of the Preferred Embodiments
The drawings will first be briefly described.

Drawings
Fig. 1A-E   is the DNA sequence for 5300 bp of genomic DNA flanking and encoding the full length DNA polymerase

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from *Thermoanaerobacter thermohydrosulfuricus* (SEQ. ID. NO. 1).

Fig. 2A-D is a contiguous open reading frame capable of encoding the full length polymerase from *Thermoanaerobacter*
5   *thermohydrosulfuricus* (SEQ. ID. NO. 2). Translation is of an open reading frame spanning nucleotides 1056-3674 of genomic DNA in Fig. 1A-E, encoding native polymerase.

Fig. 3 is the contiguous open reading frame of nucleotides 796-2616, amino acids 266-872 plus an initiator
10   methionine at the amino terminus, the exo⁻ mutant of the enzyme containing 608 amino acids (SEQ. ID. NO. 3).

Fig. 4A-C is the contiguous open reading frame of the F412Y mutation of the exo-mutant polymerase, containing 578 amino acids with. (SEQ. ID. NO. 4). At amino acid 412 there
15   is a tyrosine replacing phenylalanine.

Fig. 5 is a schematic of cloned polymerases. Letters A-D represent start sites of the various constructs shown in the table in Example 2.

Fig. 6 is a graphical representation comparing relative
20   RT activity of the exo- mutant enzyme and other enzymes that have reverse transcription activity. The x-axis indicates the polymerase (MMLV, AMV, Tth, USB). The y-axis represents reverse transcriptase activity in cpm.

Fig. 7A-C is the DNA sequence encoding a full length
25   native   polymerase   from   *Thermoanaerobacter*
thermohydrosulfuricus (SEQ. ID. NO. 23).

<u>Examples</u>

The following examples serve to illustrate the DNA
30   polymerases of the present invention and are not intended to be limiting.

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Example 1:   General Methods Used During Purification and
Characterization

Assay of DNA Polymerase Activity

5   DNA polymerase activity was assayed by measuring the
amount of incorporation of a radiolabelled deoxynucleotide
into acid percipitable material using activated salmon sperm
DNA as a template (Richardson, C. C. (1966) DNA polymerase
from *Escherichia coli*, pp. 263-276 *In* G. L. Cantoni and D.

10   R. Davies (ed.), Procedures in nucleic acid research. Harper
and Row, New York). A unit of DNA polymerase is the amount
of enzyme that catalyzes incorporation of 10 nmoles of
deoxynucleotide triphosphate into acid-precipitable material
in 30 min at 70 °C. An assay consists of 2-10 µL of protein

15   solution containing DNA polymerase being incubated with 50
µL of assay mix (20 mM Tris HCl pH 8.5 @ room temperature,
200 µM each deoxynucleotide triphosphate, 10 mM KCl, 5 mM
$MgCl_2$, 0.2 mg/mL activated salmon sperm DNA, 1 µCi 3000
Ci/mmole [α-^{32}P]dATP) at 70 °C for 10 min. The reaction is

20   stopped by adding the contents of the assay to a tube
containing 1 mL each of carrier (50 µg/mL fish sperm DNA, 2
mM EDTA) and precipitant (20% (w/v) trichloroacetic acid, 2%
(w/v) sodium pyrophosphate). After incubation on ice for at
least 5 min, the solution is filtered through a glass fiber

25   filter (e.g. GF/B, Whatman), washed with several mL ice-cold
wash solution (1 N hydrochloric acid, 100 mM sodium
pyrophosphate), placed in a counting vial with aqueous
liquid scintillation cocktail (e.g. Biosafe II, Research
Products International Corp.) and counted in a liquid

30   scintillation counter. DNA polymerase activity of the test
solution is calculated from the measured specific activity
of the assay mix. Only activity values measured to be within
the linear range of 0.01 to 0.2 units per assay reaction
were accepted as significant.

35

Measurement of Protein Concentration

Solution protein concentrations are measured spectrophotometrically by determining the absorbance of the test solution at a wavelength of 205 nm (A_{205}) (Scopes, R. K.
5   (1994) pp. 46-48 Protein Purification. Springer-Verlag, New York). The extinction coefficient of polypeptides is taken as E_{205}(1 mg/mL) = 31.

Assay of Exonuclease Activity

10   Exonuclease activity was measured as described (Kong, H., Kucera, R. B. and Jack, W. E. (1993) *J. Biol. Chem.* 268, 1965-1975) using, as substrate, *Hind*III digested phage λ DNA labeled with ^{3}H by the action of CpG methylase or a 500 bp PCR product generated from pBR322 labeled with ^{3}H by
15   incorporation of tritiated TTP. Exonuclease assays were performed in the same buffer (20 mM Tris·HCl pH 8.5 @ room temperature, 10 mM KCl, 5 mM MgCl$_2$) and at the same temperature (70 °C) as polymerase assays.

Assay for Directionality of Exonuclease

20   The directionality of the associated exonuclease activity was determined by monitoring the time-dependent release of radioactivity from a DNA substrate differentially labeled on the 5' and 3' ends as described (Kong et al., supra).

25   ## Assay for Strand Displacement Activity

An extension assay has been developed to evaluate polymerases for their utility in the SDA (strand displacement amplification) reaction (Becton-Dickinson). This extension reaction tests the ability of the polymerase
30   to displace a downstream strand and its ability to initiate extension at a nick site. This displacement and initiation at a nick is staged by annealing two primers labeled at their 5' ends immediately adjacent to each other. If the

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polymerase has strand displacement activity and is able to initiate at a nick, both primers are extended and two extension products can be visualized. If the polymerase cannot initiate at a nick (that is, it has a preference for initiation at a gap) or it lacks displacement activity then only the downstream primer is extended and only one product is detected.

Using the plasmid pBR322 as the target, two primers pBR1 and pBR2 were synthesized that anneal immediately adjacent to each other on the plasmid. Primer pBR1 is the upstream primer and corresponds to bases 3661 to 3690 in pBR322 (GGTTCCCAACGATCAAGGCGAGTTACATGA)(SEQ. ID. NO. 5). Primer pBR2 anneals downstream and corresponds to pBR322 3691-3720 (TCCCCCATGTTG TGCAAAAAAGCGGTTAGC)(SEQ. ID. NO. 6). These 30 mer oligos were purified by electroelution from a denaturing polyacrylamide gel. The primers are labeled in a kinase reaction using 10 µM primer, 50 mM Tris·HCl pH 7.5, 10 mM $MgCl_2$, 70 µCi [γ-^{32}P] ATP and 10 units T4 polynucleotide kinase for 30 min at 37 °C. A final concentration of 0.2 µM (each) of these two labeled primers are combined with 200 ng of *PstI/HincII* digested pBR322. These are denatured at 98 °C for 3 min in a buffer containing 25 mM potassium phosphate, pH 7.4, 2-8 mM $MgCl_2$, 0.2 to 1 mM dNTPs and cooled to the reaction temperature (50-70 °C) for 2 min. One unit of the polymerase is added and the reaction is continued for 10 min. The reactions are stopped by addition of an equal volume of 95% formamide, 50 mM EDTA, bromophenol blue dye. Twenty-five µL are electrophoresed over a 6% denaturing gel and the gel is exposed to autoradiographic film.

Example 2:   Purification of Native Enzyme

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Crude Purification

Growth of Thermoanaerobacter thermohydrosulfuricus Cells

A lyophilized culture of *Thermoanaerobacter thermohydrosulfuricus* strain JW102 was purchased from the
5 Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH (DSM). The growth medium used was Clostridium Thermohydrosulfuricum Medium (medium #61 in the DSM catalog of strains, 1993 edition) which consists of (per L): 10 g tryptone, 10 g sucrose, 2 g yeast extract, 0.2 g $FeSO_4 7H_2O$,
10 0.2 g $Na_2 SO_3$, 0.08 g $Na_2S_2O_3 \cdot 5H_2O$ and 1 mg Resazurin. The culture was grown at 70 °C under anaerobic conditions. After growth, the media was cooled to approximately room temperature and the culture harvested by continuous flow centrifugation. Cell paste was stored at -80 °C prior to
15 subsequent procedures.

Preparation of Extract

Frozen cell paste (5 grams) was combined with 100 mL of buffer A (50 mM Tris·HCl pH 8.0, 10 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% (w/v) glycerol). The paste was stirred at room
20 temperature until resuspension was complete. The resulting cell suspension was cooled to 0 °C by incubation in an ice bath and subjected to approximately 5 min of sonication to lyse the cells. Cell debris was removed by centrifugation at 70,000 x g for 20 min at 4 °C. The supernatant cleared
25 lysate was designated fraction 1.

Liquid Chromatography Purification

Fraction 1 was loaded onto a low-pressure liquid chromatography column (70 mL bed volume, 26 mm Ø) of DEAE cellulose (DE-52, Whatman) equilibrated with buffer A. The
30 column was washed with several column volumes of buffer A and the DNA polymerase eluted with a linear salt gradient from 10 mM to 1000 mM NaCl in buffer A at a flow rate of 0.9 mL/min. This and all subsequent chromatography steps were

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carried out at ambient room temperature (approximately 25 °C). The peak of activity eluted at approximately 32 mM NaCl. Active fractions were pooled and designated fraction 2.

5   Fraction 2 was loaded directly onto a column (22 mL bed volume, 16 mm Ø) of heparin sepharose (Heparin Sepharose CL-6B, Pharmacia) equilibrated with buffer A. The column was washed with several column volumes of buffer A and the DNA polymerase activity eluted with a linear salt gradient
10   from 10 mM to 700 mM NaCl in buffer A at a flow rate of 0.4 mL/min. The peak of activity eluted at approximately 400 mM NaCl. Active fractions were pooled and designated fraction 3.

Fraction 3 was loaded directly onto a column (2 mL bed
15   volume, 16 mm Ø) of hydroxylapatite (BioGel HA, Bio-Rad) equilibrated with buffer B (50 mM potassium phosphate, pH 7.4, 1 mM DTT, 10% (w/v) glycerol). The column was washed with several column volumes of buffer B and the DNA polymerase eluted with a linear gradient from 50 mM to 700
20   mM potassium phosphate in buffer A at a flow rate of 0.4 mL/min. The DNA polymerase containing fractions were pooled and concentrated by ultrafiltration (Centricon-50, Amicon) to a volume of 100 mL to which 100 mL of 100% glycerol was added to obtain the final product. This final preparation
25   was determined to contain about 1000 units of DNA polymerase which was judged to be approximately 80% pure on a Coomassie stained denaturing polyacrylamide gel (SDS-PAGE).

Optimized Purification

30   Preparation of Extract
Frozen cell paste (45 grams) was resuspended in 250 mL of lysis buffer (50 mM Tris-HCl, pH 8, 10 mM NaCl, 5 mM EDTA, 0.2% (v/v) NP40, 1 mM DTT). Lysozyme was added to 200 mg/mL final concentration and the solution was stirred at

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room temperature for 60 min. DNase I was added to 10 mg/mL
final concentration and incubation was continued for 10 min.
Insoluble material was removed by ultracentrifugation at
70,000 x g for 40 min. The decanted supernatant is
5   designated fraction I.

Polyethyleneimine Precipitation

A pilot polyethyleneimine (PEI) precipitation
experiment was done to determine the final PEI concentration
for this lysate to precipitate all polymerase activity with
10   the least coprecipitating protein content. PEI was added
from a 10% (v/v) stock to Fraction I to 0.4% final
concentration. The solution was stirred for 20 min at 4 °C.
The PEI precipitate was collected by centrifugation at 8,000
x g for 20 min. The polymerase activity was extracted from
15   the pellet with 150 mL of 300 mM NaCl in buffer A (50 mM
Tris-HCl, pH 8, 1 mM DTT, 1 mM EDTA and 10% glycerol). This
solution was centrifuged again at 17,000 x g. Ammonium
sulfate was added to the supernatant to 80% saturation and
the solution was stirred for one hour at 4 °C to precipitate
20   proteins from residual PEI. The ammonium sulfate fraction
was centrifuged at 48,000 x g for 30 min and the pellet
resuspended in 400 mL 10 mM NaCl, 50 mM Tri•HCl, pH 8. This
is designated Fraction II.

Liquid Chromatography Purification

25   A column (150 mL bed volume) of Heparin-Seharose CL-6B
(Pharmacia) was equilibrated with 150 mM NaCl in buffer A.
Fraction II was loaded at 150 mL/h and washed with two bed
volumes of buffer A. The polymerase activity was then
eluted with a linear gradient of 150 mM to 700 mM NaCl in
30   buffer A yielding a polymerase peak at about 400 mM NaCl.
The peak fractions were pooled and diluted fivefold with 100
mM NaCl in buffer B (50 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 1
mM EDTA and 5% glycerol). This pool is designated Fraction
III.

25

A column (4 mL bed volume) of ssDNA agarose (Pharmacia) was equilibrated with 100 mM NaCl in buffer B. One-third of the Fraction III was loaded at a flow rate of 12 mL/hr. The column was washed with the same buffer and

5   polymerase activity eluted with a linear gradient of 100 mM to 1000 mM NaCl in buffer B. The polymerase activity peak eluted at approximately 375 mM NaCl. The peak fractions were pooled and concentrated with Centricon-50 (Amicon) and stored by adding an equal volume of 100% glycerol.

10

Preparation of exo-Enzyme by Subtilisin Cleavage

Approximately 850 units of purified native *Thermoanaerobacter thermohydrosulfuricus* DNA polymerase I was exchanged into 150 mM potassium phosphate pH 6.5 by

15   three rounds of dilution in this buffer and concentration by ultrafiltration yielding a solution containing about 250 mg of protein in 550 µL. Subtilisin (10 µL of 0.06 mg/mL solution) was added to the polymerase and the mixture was incubated at 37 °C for 1 hour. After incubation, the

20   reaction was diluted to 10 mL final volume with buffer A and loaded onto a column of heparin sepharose (4 mL bed volume, 16 mm Ø) equilibrated in buffer A containing 150 mM NaCl. The column was washed with several column volumes of this buffer and the polymerase activity eluted with a linear

25   gradient of 150 mM to 700 mM NaCl in buffer A. Active fractions were pooled.

Example 3:   Characterization of Native Enzyme

General properties

The native enzyme migrates as an approximately 90,000

30   Da polypeptide (just below phosphorylase B) on SDS-PAGE. The enzyme possesses strand displacement activity. It possesses a 5'-3' exonuclease activity at approximately 1/80 the level of polymerase activity (unit:unit). The

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temperature optimum of DNA synthesis is near 75 °C under assay conditions. The optimum Mg^{2+} concentration of DNA synthesis is 1 mM. The optimum Mn^{2+} concentration of DNA synthesis is 0.5 mM.

5 **Assay for thermal stability**

To assess the thermal stability of the enzyme, a sample of native *Thermoanaerobacter thermohydrosulfuricus* DNA polymerase was incubated at elevated temperatures in several buffers. The buffers all consisted of 25 mM Tris-HCl, pH 10 8.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5% (w/v) Nonidet P-40 and 100 mg/mL BSA with the following additions:
BSA buffer : no additions.

GLY buffer : add glycerol to 40% (v/v)

15 MMO buffer : add N-methylmorpholine-N-oxide (MMO) to 3 $\underline{M}$

TMANO buffer : add Trimethylamine-N-oxide (TMANO) to 4 $\underline{M}$

Native enzyme (0.02 units/μl) was incubated in these buffers at various temperatures. At 5, 10 and 15 min, 5 μL aliquots were withdrawn and placed into a fresh tube on ice. 20 When all samples had been acquired, the heated aliquots were assayed in the usual manner. Results are expressed as the fraction activity remaining at a given time point at a given temperature.

The enzyme was least stable in BSA buffer, retaining 25 half the initial measured polymerase activity after about 5 min at 80 °C. In GLY buffer, the enzyme retained half the initial activity after about 5 min at 85 °C. In MMO and TMANO buffers, incubation at 85 to 90 °C was required to eliminate half the initial measured activity in 5 min. Incubation in 30 any of the these buffers at 75 °C for 15 min resulted in not more than 20% loss of initial DNA polymerase activity.

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Example 4:   Characterization of Subtilisin-Cleaved Enzyme

General properties

    The cleaved enzyme migrates as a doublet consisting of polypeptides migrating at approximately 64 kDa and 55 kDa on
5   SDS-PAGE. It is free of detectable exonuclease activity. The enzyme possesses strand displacement activity. The enzyme has similar thermal stability to the native enzyme.

Example 5:   Preparation of Cloned 64 kDa Enzyme

10   Preparation of genomic DNA

    Frozen cell paste of *Thermoanaerobacter thermohydrosulfuricus* (approximately 350 mg) was resuspended in 700 μL of lysis buffer (50 mM Tris·HCl pH 8.0, 50 mM EDTA, 3% SDS (w/v), 1% 2-mercaptoethanol (v/v))
15   and incubated for 1 hour at 65 °C. This solution was extracted three times with 25:24:1 phenol:chloroform:isoamyl alcohol, twice with chloroform and precipitated with two volumes of 100% ethanol. The pellet was dried briefly *in vacuo* and dissolved in 700 μL TE (10 mM Tris-HCl, pH 8.0, 1
20   mM EDTA). The concentration of genomic DNA was determined spectrophotometrically by measuring the absorbance at 260 nm $(1A_{260}=$ 50 μg/mL) and by comparison of UV fluorescence of bands on an ethidium bromide stained agarose gel relative to standard concentration markers.

25   Preparation of DNA polymerase I specific probe

    Genomic DNA was used as a substrate for a PCR reaction using degenerate oligonucleotides designed against conserved regions of DNA polymerase I family polymerases (Uemori, T., Ishino, Y., Fujita, K., Asada, K. and Kato, I. (1993) *J.*
30   *Biochem.* 113, 401-410). A standard Taq PCR reaction (20 μL) contained about 1 ng genomic DNA and 1mM of each primer EPOLF   (GACCC(ATC)AAC(CT)T(CG)CA(AG)A   A(CT)AT(ATC)CC

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(SEQ.ID.NO. 7) and EPOLR   ((GT)A(CG)(CG)A(GT) (CT)TC (AG)
TCGTG(GATC)AC(CT)TG)   (SEQ. ID. NO.   8). After one round
of PCR, the product was resolved on an agarose gel. The
product around the expected 600 bp region was excised from
5   the gel, crushed, soaked in TE and used as substrate for a
second round of PCR using the same conditions.   The
resulting ca. 600 bp product was directly sequenced using
the EPOLF and EPOLR primers (Sequenase PCR Product
Sequencing Kit, Amersham). One open reading frame was found
10 to be homologous to the targeted region of DNA polymerases I
of known sequence as judged by BLAST analysis.

Preparation of subgenomic libraries and screening

       This PCR product was labeled using a radioactive or
non-radioactive hybridization kit (Gene Images, USB) and
15 used to probe blots of agarose gels of genomic DNA digested
with various restriction enzymes. Digestion of genomic DNA
with HindIII produced a unique hybridizing fragment of
approximately 1.7 kb. DNA fragments of approximately 1.7 kb
were excised from an agarose gel and cloned into pBluescript
20 II KS (+) (Stratagene). Individual colonies that hybridized
to the probe were purified and sequenced. Similarly, double
digestion   of   Thermoanaerobacter   thermohydrosulfuricus
genomic DNA with EcoRI and XhoI produced a unique
hybridizing fragment of approximately 1.9 kb and digestion
25 with EcoRI produced a unique hybridizing fragment of
approximately 3 kb.   The sequences of these clones were
merged and formed a coherent open reading frame which had
high homology to the C-terminal portion DNA polymerases I of
known sequence as judged by BLAST analysis.   The clone
30 containing the 1.7 kb HindIII fragment was digested with
EcoRI and XhoI to produce a 1.4 kb fragment which was gel
purified, ^{32}P labeled by nick-translation and used to probe a
blot of Thermoanaerobacter thermohydrosulfuricus genomic DNA
digested with PstI and XhoI.   This identified a unique
35 hybridizing fragment of 3 kb that contained the remaining

N-terminal portion of the gene. The nucleotide sequence is presented in Fig. 1A-E (SEQ. ID. NO. 1) and a contiguous open reading frame potentially encoding the native polymerase is presented in Fig. 2A-D (SEQ. ID. NO. 2).

5   Preparation of expression vectors

Several PCR products were made from the genomic DNA that allowed cloning portions of polymerase coding sequence not including 5'-3' exonuclease domain (See Fig. 5). Letters A-D represent start sites of the various constructs
10   outlined in the table below. These truncated polymerase coding sequences were placed under the control of the λ P$_L$ promoter of the expression vector pRE2 (Reddy, P., Peterkofsky, A. and McKenney, K. (1989) *Nucleic Acids Res.* 17, 10473- 10488). These vectors were propagated in *E. coli*
15   strain DH-1 λ+ (λ cI +).

To minimize errors introduced by Taq DNA polymerase in PCR, a modified long PCR was carried out. The PCR reaction contained: 20 mM Tricine (pH 8.8), 85 mM KOAc, 200 mM each dNTP, 5% DMSO, 0.5 mM each primer, 1.5 mM MgOAc (added as
20   Hot-Start), 2.5 units Hot Tub (or rTth) DNA polymerase per 100 mL reaction (Amersham), 0.025 U Deep Vent DNA (New England Biolabs) polymerase per 100 μL reaction, 20-100 ng *Thermoanaerobacter thermohydrosulfuricus* genomic DNA per 100 μL reaction. The cycling parameters consisted of: 94 °C 30s,
25   then 68 °C 10 m 40 s x 8 cycles; 94 °C 30 s, then 68 °C 12m 00 s x 8 cycles; 94 °C 30s, then 68 °C 13 m 20 s x 8 cycles; 94 °C 30 s, then 68 °C 14 m 40 s x 8 cycles.

All PCR products were generated with the reverse primer DDREV(GGGGTACCT CTAGATCACTTGGCCAAAAACCA)   (SEQ. ID. NO. 9)
30   at the 3' end and various primers utilized at the 5' end:

DDMET (GGAATTCCATATGGCTTACCTGCTGAACCCGTCTGAGTCTAAC) (SEQ. ID. NO. 10);

DD55kd (GGAATTCCATATGGCTCTTTCTTATAAAGGAATAGAG) (SEQ. ID. NO.
35   11);

30

DD64kd (GGAATTCCATATGAAAGTTGAAAAATGGTCA) (SEQ. ID. NO. 12);

DD64pref (GGAATTCCATATGAAAGTTGAAAAATGGTCTAAAGTAGACATCAAAGAATT
AG) (SEQ.ID.NO.13);

DDHF (GGAATTCCATATGAAGCTTTTAGAGCTTTT) (SEQ. ID. NO. 14);

5   DD67kd (GGAATTCCATATGAAACTTTTAGAGCTTTTCAACAAATTAGAATTC)
(SEQ. ID. NO. 15).

        The resultant PCR products were digested with *Nde*I and
*Kpn*I and cloned into pRE2 digested with the same restriction
enzymes.

10   Silent changes (see Table below) denotes that the 5'
primer encodes some codons which are synonymous to the
native sequences, but are more frequently utilized by *E.
coli*. By this method, six different clones were constructed
which encode four different polypeptides.

Site	Plasmid	MW	codons	5' Primer	Note
D	pMET11	55 kDa	483	DDMET	Silent changes, native MET
C	pRED5504	58 kDa	502	DD55kd	Native sequence + MET
B	pRED6404	64 kDa	579	DD64kd	Native sequence + MET
B	pPREF2	64 kDa	579	DD64pref	Silent changes + MET
A	pRED4	67 kDa	609	DDHF	Native sequence + MET
A	pRED6706	67 kDa	609	DD67kd	Silent changes + MET

Expression of Cloned 64 kDa Enzyme

5   *E. coli* strain MZ-1 (cI857 lysogen) harboring the plasmid pRED6404 was cultured in LB medium (per L: 10 g tryptone, 5 g yeast extract, 10 g NaCl) supplemented with 50 µg/mL ampicillin. The culture was grown at 30-32 °C until the OD_{600} reached 1.5 at which time the culture temperature was

10 raised to 40-42 °C. Growth continued at this temperature for 2 hours before the culture was harvested in a continuous flow centrifuge. Cell paste was stored at -80 °C prior to subsequent procedures.

Example 6:   Preparation of Cloned Full-length Enzyme

15   Full length enzyme was cloned by PCR from the clone of genomic DNA (SEQ. ID. NO. 1) (Fig. 1A-E). A 5' primer DDF-FOR (GGAATTCCATATGGCTTATAAATTTTTAATCATTGATGGTAGTAGC) (SEQ.

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ID.NO. 24) was synthesized. This primer encodes an *Nde* I
site, inserts an alanine codon after the initiating
methionine and changes two codons to synonymous codons
favored by *E. coli* (ATA -> ATC, Ile at aa position 6 and GGA
5   -> GGT, Gly at aa position 9). This primer was used in
conjunction with a vector primer from the assembled clone of
genomic DNA to produce a PCR product which was subsequently
digested with *Nde* I and *Xho* I and subcloned into similarly
digested pRED6404 to form pLS-3. The DNA for the full
10   length enzyme is shown in Fig. 7 A-C (SEQ. ID. NO. 23).

Example 7:   Purification of Cloned 64 kDa Enzyme

Preparation of Extract
        Frozen cell paste (370 grams) was combined with 1800 mL
of buffer A (50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM DTT, 1
15   mM EDTA, 10% (w/v) glycerol). The paste was stirred at room
temperature until resuspension was complete. The cells were
lysed using a French pressure cell. Cell debris was removed
by centrifugation at 70,000 x g for 20 min at 4 °C.

Liquid chromatography purification
20   The cleared lysate was passed through a column of DEAE
cellulose at 300 mM salt. The collected flow through was
heated to 70 °C for 10 min, cleared again by centrifugation
and loaded onto a column heparin sepharose. The DNA
polymerase was eluted from this column with a salt gradient
25   as before and was found in most cases to be pure. If not,
column chromatography on hydroxylapatite was performed.

Example 8:   Characterization of Cloned 64 kDa Enzyme

General properties
        Cloned enzyme refers to the purified gene product of
30   pRED6404. The cloned enzyme migrates as an approximately
64,000 Da polypeptide (just below bovine serum albumin, MW

67,000) on SDS-PAGE. The specific activity of the cloned
enzyme is 28,000 units/mg. It is free of detectable
exonuclease activity. The temperature optimum of DNA
synthesis is near 72 °C under assay conditions. The optimum

5   Mg^{2+} concentration of DNA synthesis is 2 mM. The optimum Mn^{2+}
concentration of DNA synthesis is 0.7 mM.

Reverse transcriptase activity

      The cloned enzyme functioned as a reverse transcriptase
in an assay containing poly(rA):oligo(dT_{12-18}) and [^{3}H]TTP at

10   42 °C. This enzyme has RT activity in the presence of Mg^{2+}
with or without the addition of Mn^{2+}.

Measurement of reverse transcriptase activities of different
enzymes using an SPA format

      Amersham reverse transcriptase scintillation proximity

15   assay (SPA) kit (NK9020), and MMLV (E 70456), and AMV (E
70041) enzymes were supplied by Amersham International. The
cloned 64 kD enzyme prepared above was designated as USB
enzyme.   Tth   (N808-0098)   enzyme   was   obtained   from
Perkin-Elmer.

20   MMLV and AMV enzymes were tested at 37 °C and 42 °C
respectively in the presence of magnesium. The Tth and USB
enzymes were both used at 60 °C with manganese present as the
co-factor. The assay buffer used was the same as supplied
in   the   SPA   kit   except   the   appropriate   co-factor   was

25   substituted in the buffer mix. Essentially, the assay was
set up as follows in duplicate using the protocol provided
in the SPA kit in sarstedt tubes in a total reaction volume
of 100 μL.

      80 μL of 0.77 μM [^{3}H]TTP in a buffer consisting of:

30

                          50 mM Tris·HCl pH 8
                          80 mM KCl
                          10 mM $MgCl_2$ or 4 mM $MnCl_2$
                          10 mM DTT

34

0.05% w/v NP40

10 µL of 0.2 µM primer/template (biotinylated (dT$_{16}$) oligonucleotide annealed to poly r(A)) in

10 mM Tris·HCl pH 8

5   1 mM MgCl$_2$

10 µL of 1 unit (as defined by the manufacturer) of enzyme diluted in 50 mM Tris-HCl, pH 8, 10 mM DTT was added to each tube to start the reaction and incubated at the indicated temperature for 30 min after which the reaction
10 was terminated with the addition of 10 µL 0.56 M EDTA, pH 8. 10 µL of the supplied streptavidin SPA beads were then added to each tube, and after incubating for a further 10 min at room temperature, were counted in a LKB 1205 scintillation counter.

15   Appropriate controls without enzyme were also performed. The data obtained is shown in Fig. 6. This experiment does not take into account the melting temperature of the primer used in this experiment which at 60 °C would theoretically leave only a minority of the primer
20 still being annealed to the template. This may actually result in an underestimate of the activity observed with the USB and Tth enzymes.

Use of 64 kDa polymerase in Strand Displacement
Amplification

25   The exonuclease deficient cloned enzyme described above was used in a Strand Displacement Amplification (SDA) reaction (Walker et al., (1992) Proc. Natl. Acad. Sci. USA 89:392-396). The target DNA to be amplified is a 1.2 kb clone of the insertion element IS 6110 from Mycobacterium
30 tuberculosis [GenBank accession no. M29899]. The target DNA is cloned into pBluescript KS (Stratagene, La Jolla, CA). The reaction mixture (final concentrations: 25mM potassium phosphate, pH 7.4, 0.1 µg/mL BSA, 0.05 µM each primers B1 (CGATCGAGCAAGCCA) (SEQ. ID. NO. 16) and B2 (CGAGCCGCTCGCTGA)
35 (SEQ. ID. NO. 17), 0.5 µM each primers S1.1

35

(ACCGCATCGAATGCATGTCTCGGTAGGCG ACTCGACC) (SEQ. ID. NO. 18)
and S2.2 (CGATTCCGCTCAGACTTCTCGGGT TACTGAGATCCCT) (SEQ. ID.
NO. 19), 1.4 mM dCTPαS, 0.5 mM each dATP, dGTP and dCTP and
10^7 copies of target plasmid) was heated to 100 °C for 3 min
5   to denature the target and then placed at 55 °C. After about
5 min at 55 °C, enzyme mix was added (final concentrations:
35 Units 64 kD polymerase, 160 Units *Bso*BI restriction
enzyme (New England Biolabs, Beverly, MA) and 6.9 mM MgCl₂
(total reaction volume 50 μL) and incubation was continued
10   for 10 min.   An equal volume of stop solution (95%
formamide, 20 mM EDTA and 0.05% each bromophenol blue and
xylene cyanol FF) was added and 5 μL of this was
electrophoresed on a 10% denaturing polyacrylamide gel. DNA
was visualized by staining with ethidium bromide. The 64
15   kDa polymerase produced the expected 105 nucleotide full
length product (major product) and the expected 86
nucleotide nicked product (minor product).

Use of 64 kDa polymerase in RT-PCR
         The 64   kD polymerase was used as a reverse
20   transcriptase in conjunction with AmpliTaq in RT-PCR. Using
rabbit globin mRNA as a template, 10^6 copies were detectable
under the following protocol. In a reaction volume of 100
μl, the following components were assembled: 1X PCR buffer
(10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2 mM MgCl₂, 200 μM each
25   dNTP, 100 U human placental RNAse inhibitor (Amersham), 10^6
copies rabbit globin mRNA (BRL), 30 pmoles globin-down
primer   (TCAGTGGTATTTGTGAGCCAGGGCATTGGCCACACCAGCCACCACCTTCT)
(SEQ. ID. NO. 25), 15 pmole globin-up primer
(ATCCCCCAAAACAGACAGAATGGTGCATCTGTCC) (SEQ. ID. NO. 26), 2.5
30   U AmpliTaq polymerase and 5 U 64 kD polymerase. The
reaction was incubated at 65 °C for 30 min followed by 5 min
at 95 °C, 35 cycles of (95 °C for 1 min, 65 °C for 1 min, 70
°C for 2 min) followed by 72 °C for 5 min. The product was
resolved on a 2% ethidium bromide stained agarose gel and
35   the expected 463 bp product was observed.

36

Using total RNA from rat liver, an RT-PCR product from the albumin gene was detected from 250 ng of template using the following protocol. In a reaction volume of 100 μL, the following components were assembled: 1X PCR buffer (10 mM
5   Tris-HCl, pH 8.3, 50 mM KCl), 2 mM MgCl₂, 200 μM each dNTP, 100 U human placental RNAse inhibitor (Amersham), 250 ng rat liver RNA, 30 pmoles albumin-down primer (GTTCTCCTGGAAGGAGGTGCACATGGCCTC) (SEQ. ID. NO. 27), 15 pmole albumin-up primer (CACACAAGAGTGAGATCGCCCATCGGTTTAAGGA) (SEQ.
10 ID. NO. 28), 2.5 U AmpliTaq polymerase and 40 U 64 kD polymerase. The reaction was incubated at 65 °C for 30 min followed by 5 min at 95 °C, 35 cycles of (95 °C for 1 min, 65 °C for 1 min, 70 °C for 2 min) followed by 72 °C for 5 min. The product was resolved on a 2% ethidium bromide stained
15 agarose gel and the expected 386 bp product was observed.

Example 9:   Construction of FY DNA polymerase from
Thermoanaerobacter thermohydrosulfuricus

Starting Material

DNA constructs that were used to generate the FY
20 polymerase were as follows:

pRED6706 is an expression vector which encodes a polypeptide of 608 amino acids. After an engineered initiating methionine, the remaining 607 amino acids encoded represent the C-terminal portion of the DNA polymerase I
25 obtainable from of Thermoanaerobacter thermohydrosulfuricus. pRED6404 is an expression vector which encodes a polypeptide of 578 amino acids. After an engineered initiating methionine, the remaining 577 amino acids encoded represent the C-terminal portion of the DNA polymerase I obtainable
30 from Thermoanaerobacter thermohydrosulfuricus.

pPREF2 encodes the same polypeptide as pRED6404, but has three silent mutations of the Thermoanaerobacter thermohydrosulfuricus sequence at codons 7, 10 and 11 to eliminate codons rarely utilized by E. coli.

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All of these constructs are derived from expression vector pRE2 (*Nucleic Acids Res.* (1989) 17, 10473-88) which utilizes the λ P_L promoter and λ cII ribosome binding site.

Construction of F412Y

5   *Thermoanaerobacter thermohydrosulfuricus* DNA polymerase was searched for the sequence (RXXXKXXXFXXXYG) (SEQ. ID. NO. 20) to locate the motif B or O-helix region. The sequence (RRAAKAVNFGIIYG) (SEQ. ID. NO. 21) was found at amino acid residues numbered 433 through 466 of the predicted pRED6706

10   gene   product.   A   mutagenic   oligonucleotide, DDFY (AAGGTCAGAAAGCCCATAATCGCTTATGCCATATATTATG CCATAATTTACGGC) (SEQ. ID. NO. 22) was designed anti parallel to nucleotides 1315-1371 of the pRED6706 coding sequence except for the change (AAA-ATA) at nt 1325. This change

15   corresponds to phenylalanine being changed to tyrosine in the expressed polymerase. This oligonucleotide also overlaps a unique *Xho*l restriction site at nucleotide 1361.

Oligonucleotide DDFY together with DD64pref (SEQ. ID. NO. 13) which is complementary to nucleotides 94-133 of the

20   pRED6706 coding sequence, contains an *Nde*I restriction site, were used to direct PCR amplification and simultaneous mutagenesis of pRED6706. The resulting PCR product was digested with *Nde*I and *Xho*I, purified by agarose gel electrophoresis and ligated with the large fragment of

25   similarly treated pRED6404. The ligated construct was used to transform competent *E. coli* strain DH1 λ+ (λcI +). Clones yielding an appropriate restriction enzyme digestion pattern were sequenced to confirm the mutation. Spurious mutations were found in addition to the desired mutation in

30   all clones examined, so an *Afl*III/*Kpn*I restriction fragment containing   exclusively   the   F412Y   mutation   (pRED6404 numbering) was subcloned in pRED6404 to yield pLS-1.

<u>Example 10:</u>   <u>Purification of F412Y DNA polymerase</u>

Plasmid pLS-1 was transformed into *E. coli* strain MZ-1
(λ   cI857 lysogen).   One L of culture in LB medium
containing 100 µg/mL Ampicillin was grown at 30 °C until OD$_{600}$

5 = 1.1.   The temperature was raised to 42 °C and growth
continued for 2 hours. The cells were harvested by
centrifugation (4 g wet weight) and resuspended in 15 mL
buffer A (50 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM PMSF, 1 mM
EDTA, 10% (v/v) glycerol) containing 50 mM NaCl.   The

10 resuspension was sonicated four times for 30 seconds and the
lysate was cleared by centrifugation.   The cleared lysate
was made 300 mM in NaCl and passed through a column of DEAE
cellulose (65 mL bed volume) equilibrated in buffer A
containing 300 mM NaCl.   The flow through fraction (45 mL)

15 was collected, heated to 70 °C for 15 min and cleared by
centrifugation.   The cleared supernatant was diluted with
buffer A containing 50 mM NaCl to a total volume of 120 mL.
This solution was loaded onto a column of heparin sepharose
(70 mL bed volume) equilibrated with buffer A containing 150

20 mM NaCl.   The column was washed with this same buffer and
the activity eluted from the column with a linear salt
gradient from 150 mM to 700 mM NaCl in buffer A.   Active
fractions judged to be greater than 90% pure by SDS-PAGE
were pooled, concentrated by ultrafiltration and dialyzed

25 against 50 mM phosphate 50% (v/v) glycerol. The sequence is
provided in Fig. 4A-C (SEQ. ID. NO. 4).

<u>Example 11:</u>   <u>Full Length 5' to 3' Exonuclease Deficient</u>
<u>Mutant</u>

The removal of 5' to 3' exonuclease activity is
30 desirable for applications suitable for this polymerase,
including amplification and sequencing. Because the 5' to
3' exonuclease activity may be co-incident with RNaseH
activity, it is also important to remove or reduce the 5' to
3' exonuclease activity for the application of reverse

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transcription. Single nucleotide substititions are suitable
to reduce or remove exonuclease activity.

The amino acid sequence of Tts polymerase was compared
with known homologous DNA Polymerase I enzymes such as *E.*
5   *coli* DNA Pol I, Taq DNA polymerase and Tth DNA polymerase to
identify conserved motifs in the 5' to 3' exonuclease domain
(Gutman and Minton, <u>Nucleic Acids Research</u>, 21:4406-4407,
1993).

Tts	5	LII<u>D</u>GSS	11
Pol I	10	ILV<u>D</u>GSS	16
Taq	15	LLV<u>D</u>GHH	21
Tth	15	LLV<u>D</u>GHH	21

A carboxylate residue known to be present at the
exonuclease active site of Taq DNA polymerase (Kim et al.,
15 <u>Nature</u> 376:612-616, 1995) was mutangenized. This residue
corresponds to aspartic acid residue at position 8 (D8) in
Tts polymerase, which was mutagenized to an alanine residue
(A) to remove the carboxylate function and abolish the 5' to
3'   exonuclease   activity.   An   oligo   primer
20 (CTTACATATGGCTTATAAATTTTTAATCATTGCTGGTAGTAG) (Seq. I.D. No.
28) was used to perform the site directed mutagenesis. This
mutagenesis converts Asp^8 to Ala^8 and introduces a convenient
cloning site (NdeI) at the 5' end. The pLS-3 construct
(full length Tts polymerase) was used as template and
25 parental clone. A 1.45 kb product was amplified by PCR with
the above primer and an internal Tts reverse primer. The
NdeI/EcoRI fragment of this PCR product was substituted back
into the pLS-3 clone. Vector pRE2 and bacterial strains DH1
λ+ and M5248 are as described previously.

30   Those who practice the art can utilize known
techniques, and others known to those who practice the art,
to identify and alter other amino acid residues in the amino
terminal one third of the Tts polymerase to remove the
exonuclease activity. There are at least six conserved
35 motifs located at the amino terminus of homologous DNA
polymerases, in which changes in amino acid residues result

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in a loss or a reduction in exonuclease activity. These
conserved motifs are identified in Gutman and Minton (*supra*)
and are supported by data from the crystal structure of Taq
polymerase (Kim, *et al.* (1995) *Nature* 376:612-616). The
5   present invention encompasses alterations in these regions.

Example 12:   PCR with ΔTts in 30% Glycerol

PCR was performed with ΔTts enzyme in presence of 30%
glycerol, in order to stabilize the enzyme and to reduce the
temperatures necessary for DNA annealing in the reaction. A
10 100 µL PCR reaction contains 10 ng of a 6 kb plasmid
template, 100 pmol each of forward and reverse primer about
500 bp apart, 200 µM dNTP, 10 mM Tris-HCL, pH 8.3, 50 mM
KCL, 1.5 mM $MgCL_2$ and 30% glycerol. The reaction mix was
heated to 95 °C for 2 min before ΔTts was added. An
15 AmpliTaq™ (PE Applied Biosystems, Foster City, CA) control
reaction was performed separately under the same conditions.
The cycling program consisted of: 80 °C for 15 sec, 40 °C for
30 sec and 65 °C for 1 min, repeated for 30 cycles. A PCR
product of appropriate size was generated by 2.5, 5 and 10
20 units of ΔTts, and this amount of product was two to three
fold greater than that made by 2.5 units of AmpliTaq™ with
the same conditions.

Example 13:   RT/PCR protocol with ΔTts

Reverse transcription is performed with ΔTts enzyme, as
25 the first step of RT/PCR. A 20 µL reaction contains 40 units
of ΔTts enzyme, 5 mM $MgCL_2$, 20 units of placental Rnase
inhibitor (Amersham Pharmacia Biotech, Inc., Cleveland, OH),
1 mM each of dATP, dCTP, dGTP, and dTTP, 15 pmol primer, 10
mM Tris-HCl pH 8.3, 50 mM KCl, and 50 ng globin mRNA
30 template. The reaction is allowed to proceed for 45 min at
65 °C. This 20 µL reaction mixture is added to a PCR
reaction mixture with a final volume of 100 µL, containing
10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCL_2$, 100 pmol each
of forward and reverse primers about 500 bp apart, and 30%

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glycerol. Further addition of enzyme is not necessary; ΔTts performs PCR in the presence of glycerol. The cycling program consists of 50 cycles of: 80 °C for 15 sec, 40 °C for 30 sec, 65 °C for 1 min.

5   One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods, kits, solutions, and molecules, described herein are presently

10   representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the

15   claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

20   All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was

25   specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically

30   disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description

35   and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any

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equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

Other embodiments are within the following claims.

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Claims

1.   Method for polymerase chain reaction, in the
presence of a polymerase stabilizing agent, utilizing an
enzymatically active DNA polymerase having at least 80%
5   identity in its amino acid sequence to the DNA polymerase of
Themoanaerobacter thermohydrosulfuricus and an exonuclease
activity removed.

2.   The method of claim 1, wherein said exonuclease
10 activity is removed by having an N-terminal deletion to give
between 540 to 582 amino acids.

3.   The method of claim 1, wherein said exonuclease
activity is removed by deleting up to one third of the amino
15 acid sequence at the N-terminal.

4.   The method of claim 1, wherein said exonuclease
activity is removed by substitutition of an amino acid in
the amino terminal one third of the protein.
20

5.   The method of claim 4, wherein said substitution
is replacing the aspartic acid residue at position 8 with an
alanine residue.

25   6.   The method of claim 1, wherein said agent is
independently selected from the group consisting of
glycerol, trimethylamine-N-oxide, and N-methylmorpholine-N-
oxide.

30   7.   The method of claim 1, wherein said agent is
glycerol at a concentration of 30%.

8.   Kit for polymerase chain reaction comprising a
polymerase stabilizing agent, and an enzymatically active
35 DNA polymerase having at least 80% identity in its amino

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acid sequence to the DNA polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed.

5   9.   The kit of claim 8, wherein said exonuclease activity is removed by having an N-terminal deletion to give between 540 to 582 amino acids.

10.   The kit of claim 8, wherein said exonuclease activity is removed by deleting up to one third of the amino
10   acid sequence at the N-terminal.

11.   The kit of claim 8, wherein said exonuclease activity is removed by substitutition of an amino acid in the amino terminal one third of the protein.
15

12.   The kit of claim 8, wherein said exonuclease activity is removed by replacing the aspartic acid residue at position 8 with an alanine residue.

20   13.   The kit of claim 8, wherein said agent is independently selected from the group consisting of glycerol, trimethylamine-N-oxide, and N-methylmorpholine-N-oxide.

25   14.   The kit of claim 8, wherein said agent is glycerol at a concentration of 30%.

15.   Solution for use in polymerase chain reaction comprising a polymerase stabilizing agent, and an
30   enzymatically active DNA polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed.

45

16. The solution of claim 15, wherein said exonuclease activity is removed by having an N-terminal deletion to give between 540 to 582 amino acids.

5   17. The solution of claim 15, wherein said exonuclease activity is removed by deleting up to one third of the amino acid sequence at the N-terminal.

18.   The   solution   of   claim   15,   wherein   said
10   exonuclease activity is removed by substitutition of an amino acid in the amino terminal one third of the protein.

19. The   solution   of   claim   18,   wherein   said substitution is replacing the aspartic acid residue at
15   position 8 with an alanine residue.

20. The solution of claim 15, wherein said agent is independently   selected   from   the   group   consisting   of glycerol, trimethylamine-N-oxide, and N-methylmorpholine-N-
20   oxide.

21. The solution of claim 15, wherein said agent is glycerol at a concentration of 30%.

25   22. An enzymatically active DNA polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of *Thermoanaerobacter thermohydrosulfuricus* and having an exonuclease activity removed by substitutition of an amino acid in the amino terminal one third of the
30   protein.

23. The polymerase of claim 22, wherein said DNA polymerase has the phenylalanine at position 706 replaced with a tyrosine.

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24. An enzymatically active DNA polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of *Thermoanaerobacter thermohydrosulfuricus* and having an exonuclease activity removed by replacing the aspartic acid residue at position 8 with an alanine residue.

25. The polymerase of claim 24, wherein said DNA polymerase has the phenylalanine at position 706 replaced with a tyrosine.

26. A stable enzymatic composition comprising a purified DNA polymerase of any of claims 22, 23, 24 or 25 in a buffer.

27. Purified nucleic acid encoding the DNA polymerase of any of claims 22, 23, 24 or 25.

28. Host cell comprising a vector containing a nucleic acid sequence encoding the DNA polymerase of any of claims 22, 23, 24 or 25.

29. A DNA polymerase of any of claims 22, 23, 24 or 25 and a thermostable restriction enzyme for use in strand displacement amplification.

30. Method for generating and amplifying a nucleic acid fragment by strand displacement utilizing a DNA polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of *Thermoanaerobacter thermohydrosulfuricus* and having an exonuclease activity removed by substitutition of an amino acid in the amino terminal one third of the protein.

31. Method for generating and amplifying a nucleic acid fragment by strand displacement utilizing a DNA polymerase having at least 80% identity in its amino acid

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sequence to the DNA polymerase of *Thermoanaerobacter thermohydrosulfuricus* and having an exonuclease activity removed by replacing the aspartic acid residue at position 8 with an alanine residue.

32. Kit for strand displacement amplification of DNA comprising a DNA polymerase of any of claims 52, 53, 54 or 55 and a thermostable restriction enzyme.

33. Method for sequencing DNA whereby at least a part of the nucleotide base sequence of a DNA template can be determined comprising the step of generating chain terminated fragments from said DNA template with a DNA polymerase of claim 53 or 55 in the presence of at least one chain terminating agent and determining the sequence of at least part of said DNA template from the sizes of said fragments.

34. Kit for sequencing DNA comprising a DNA polymerase of claim 53 or 55 and a pyrophosphatase.

35. Method for preparing cDNA by providing an oligonucleotide primer, a polymerase of any of claims 22 or 24 and between one and four deoxyribonucleoside phosphates.

36. A DNA polymerase of claim 22 or 24, wherein said polymerase has a reverse transcriptase activity.

37. Method for reverse transcription/polymerase chain reaction utilizing a DNA polymerase of claim 22 or 24 and a polymerase suitable for carrying out polymerase chain reaction in a single reaction vessel.

38. The method of claim 37, wherein said polymerase suitable for carrying out polymerase chain reaction is *Thermus aquaticus* DNA polymerase.

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39. Kit for reverse transcription/polymerase chain reaction comprising a DNA polymerase of claim 22 or 24 and a polymerase suitable for carrying out polymerase chain

5   reaction.

40. The kit of claim 39, wherein said polymerase suitable for carrying out polymerase chain reaction is *Thermus_aquaticus* DNA polymerase.

10

41. Solution for use in RT/PCR comprising DNA polymerase of claim 22 or 24 and a polymerase suitable for carrying out polymerase chain reaction.

15   42. The solution of claim 41, wherein said polymerase suitable for carrying out polymerase chain reaction is *Thermus aquaticus* DNA polymerase.

43. Solution for strand displacement amplification of

20 DNA comprising a DNA polymerase of claims 22, 23, 24 or 25 and a thermostable restriction enzyme.

44. Solution for sequencing DNA comprising a DNA polymerase of claims 23 or 25 and a pyrophosphatase.

25

45. Method for reverse transcription/polymerase chain reaction, in the presence of a polymerase stabilizing agent, utilizing an enzymatically active DNA polymerase having at least 80% identity in its amino acid sequence to the DNA

30 polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed.

46. The method of claim 45, wherein said exonuclease activity is removed by having an N-terminal deletion to give

35 between 540 to 582 amino acids.

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47. The method of claim 45, wherein said exonuclease activity is removed by deleting up to one third of the amino acid sequence at the N-terminal.

5   48. The method of claim 45, wherein said exonuclease activity is removed substitutition of an amino acid in the amino terminal one third of the protein.

49. The method of claim 48, wherein said substitution
10 is replacing the aspartic acid residue at position 8 with an alanine residue.

50. The method of claim 45, wherein said agent is independently selected from the group consisting of
15 glycerol, trimethylamine-N-oxide, and N-methylmorpholine-N-oxide.

51. The method of claim 45, wherein said agent is glycerol at a concentration of 30%.
20

52. Kit for reverse transcription/polymerase chain reaction comprising a polymerase stabilizing agent and an enzymatically active DNA polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of
25 *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed.

53. The kit of claim 52, wherein said exonuclease activity is removed by having an N-terminal deletion to give
30 between 540 to 582 amino acids.

54. The kit of claim 52, wherein said exonuclease activity is removed by deleting up to one third of the amino acid sequence at the N-terminal.
35

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55. The kit of claim 52, wherein said exonuclease activity is removed by substitutition of an amino acid in the amino terminal one third of the protein.

56. The kit of claim 55, wherein said substitution is replacing the aspartic acid residue at position 8 with an alanine residue.

57. The kit of claim 55, wherein said agent is independently selected from the group consisting of glycerol, trimethylamine-N-oxide, and N-methylmorpholine-N-oxide.

58. The kit of claim 55, wherein said agent is glycerol at a concentration of 30%.

59. Solution for use in reverse transcription/polymerase chain reaction comprising a polymerase stabilizing agent and an enzymatically active DNA polymerase having at least 80% identity in its amino acid sequence to the DNA polymerase of *Themoanaerobacter thermohydrosulfuricus* and an exonuclease activity removed.

60. The solution of claim 59, wherein said exonuclease activity is removed by having an N-terminal deletion to give between 540 to 582 amino acids.

61. The solution of claim 59, wherein said exonuclease activity is removed by deleting up to one third of the amino acid sequence at the N-terminal.

62. The solution of claim 59, wherein said exonuclease activity is removed by substitutition of an amino acid in the amino terminal one third of the protein.

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63. The solution of claim 62, wherein said substitution is replacing the aspartic acid residue at position 8 with an alanine residue.

5   64. The solution of claim 59, wherein said agent is independently selected from the group consisting of glycerol, trimethylamine-N-oxide, and N-methylmorpholine-N-oxide.

10   65. The solution of claim 59, wherein said agent is 30% glycerol.

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CTGCAGAAAAAAATAATAAAAAGTTTAAAAAATATGCCTCCTATAGAACTTCCTGAAGATT

TTAATAAAAAATTACATCA

AAAATTGATTTATCATAAAAATTTTTTAGAGAAGAAAAAAGAAAGGCTAAAAAAGACGTTT

AACAATAGTAGCTATTGCG

ACATCTTTAATATTTATGACAGTATTTATAGTGAATACATTTAATAAATCCTTACAAATTG

AGTCTAATACTCCTTTAGC

TAACATTGAAATGGGACAGAGTTATGGCTTACCTCAGAAAGAAGGAGCTAATAATGAAAGA

GGATTGCCAGCAGGAACTT

CAAGGAAAATTACAAAGAATGCAACCATTTCCTTGGAAGTAGAAGACGTCAATGGATGTTA

TGATAAAGTCTTTCAGTTA

GTAAAAGAAGCAGAAGGTTTTATTGAAACTTCTGAGGAAACAGTATCCAGTGATAATATCA

AAAGGGTAAATGTTGTTTT

GAAGGTGCGGGAAGATAAATTTGAAAGTGTGATTTCTCAAATTAAAGATTTGGCAAAGTAA

CAGCTTTAAGGATTGACAG

CAAAGACATTACAGACCAGTATTATGACTTACAAGCAAGGCTAAAAAATTTGGAAGTAGAG

GAACAAAAGCTTCAAGACA

TCATGAATAAGGCTACTACAGTCAAGGAAATGCTTGAAGTAGAATCTGAAATAAACAGGAT

AAGAAGTGAAATAGAGTCA

ATGGAAGGACAGCTAAAATTATGGGAAAATCTTACGAGTCTGGCAACCATAAATCTTTCAA

TAAAAGAGATACCTAAAGA

TGAGAAACCTTTGGCTATAGTTTCTTTTAAGGGAATTGGTAAAGGTATAAAAGAAGCATTT

ATAAATAATATGAATTTTT

TAATATTTTTTATGAAAAAGTTGATAATAATATTGGTGATAATTTTGCCTTATAGTGTACT

GGCTTTAATCGGTTATAAA

GTGTATATCTATTTCAAAAAAAGGAGATGACAATTTACATCTTCTTTTTTTTGCGGTATAT

TAAAATACAAAGAAATAAA

AATGGGAGTGATTTAATGTATAAATTTTTAATAATTGATGGAAGTAGCCTCATG

Fig. 1A

TACAGAGCCTATTATGCCTTGCCCAT

GCTTACTACAAGTGAGGGATTGCCTACAAATGCTCTGTATGGTTTTACTATGATGCTTATA

AAACTTATCGAGGAGGAAA

AACCTGATTACATAGCTATTGCTTTTGACAAAAAAGCTCCTACTTTTAGACACAAAGAATA

TCAAGACTACAAAGCTACA

AGACAAGCTATGCCTGAAGAACTTGCTGAACAAGTAGACTATTTGAAAGAAATTATAGATG

GCTTTAATATAAAGACATT

AGAATTAGAAGGTTATGAAGCTGATGACATTATAGGGACTATTTCAAAGCTGGCAGAGGAA

AAAGGAATGGAAGTGCTTG

TAGTTACAGGAGACAGAGATGCTCTTCAATTAGTTTCAGATAAAGTGAAGATAAAAATTTC

TAAAAAGGGTATTACTCAG

ATGGAAGAGTTTGACGAAAAGGCTATTTTAGAAAGGTATGGAATAACTCCTCAGCAGTTTA

TAGATTTAAAAGGGCTTAT

GGGAGATAAATCTGATAATATCCCTGGAGTACCTAATATAGGGGAAAAAACTGCGATTAAG

CTATTAAAGGATTTTGGAA

CAATTGAAAATTTAATCCAAAATCTTTCTCAGCTTAAAGGTAAAATAAAAGAAAATATAGA

AAACAATAAAGAGTTAGCT

ATAATGAGTAAGAGGCTTGCTACTATAAAAAGAGACATTCCCATTGAGATAGATTTTGAGG

AGTATAAAGTAAAAAAATT

TAATGAGGAGAAGCTTTTAGAGCTTTTTAATAAATTAGAATTCTTTAGTTTAATTGATAAC

ATAAAGAAAGAAAGTAGCA

TAGAGATTGTAGATAATCATAAAGTTGAAAAATGGTCAAAAGTAGATATAAAAGAATTAGT

AACTTTGTTGCAAGATAAC

AGAAATATTGCTTTTTACCCGTTAATTTATGAAGGGGAAATAAAAAAAATAGCCTTTTCTT

TTGGAAAGGATACGGTTTA

TATTGACGTTTTCCAAACAGAAGATTTAAAGGAGATTTTTGAAAAGAAGATTTTGAATTT

ACAACCCATGAAATAAAGG

ATTTTTTAGTGAGGCTTTCTTATAAAGGAATAGAGTGTAAAAGCAAGTACATAGATACTGC

TGTAATGGCTTATCTTCTG

Fig. 1B

AATCCTTCTGAGTCTAACTATGACTTAGACCGTGTGCTAAAAAAATATTTAAAGGTAGATG

TGCCTTCTTATGAAGGAAT

ATTTGGCAAAGGTAGGGATAAAAAGAAAATTGAAGAGATTGACGAAAACATACTTGCTGAT

TATATTTGCAGTAGATGTG

TGTATCTATTTGATTTAAAAGAAAGCTGATGAATTTTATTGAAGAGATGGATATGAAAAA

ACTTCTATTAGAAATAGAA

ATGCCTCTTGTAGAAGTTTTAAAATCAATGGAGGTAAGTGGTTTTACATTGGATAAAGAAG

TTCTAAAAGAGCTTTCACA

AAAGATAGATGATAGAATAGGAGAAATACTAGATAAAATTTATAAAGAGGCAGGATATCAA

TTTAATGTAAATTCACCTA

AGCAATTAAGTGAATTTTTGTTTGAAAAGTTAAACTTACCAGTAATAAAGAAAACAAAAAC

AGGATACTCTACGGATTCT

GAAGTTTTGGAACAATTGGTTCCTTATAATGATATTGTCAGCGATATAATAGAGTATCGGC

AACTTACAAAACTTAAATC

TACTTATATAGATGGATTTTTGCCTCTTATGGATGAAAACAATAGAGTACATTCTAATTTT

AAACAAATGGTTACTGCTA

CAGGTAGAATAAGCAGCACCGAGCCAAATCTACAAAATATACCTATAAGAGAAGAGTTTGG

CAGACAAATTAGAAGGGCT

TTTATTCCGAGGAGTAGAGATGGATATATTGTTTCAGCAGATTATTCTCAGATTGAACTGA

GGGTTTTAGCACATGTTTC

GGGAGATGAAAAGCTAATAGAATCTTTTATGAATAATGAAGATATACATTTAAGGACAGCT

TCGGAGGTTTTTAAAGTTC

CTATGGAAAAAGTTACACCGGAGATGAGAAGAGCAGCAAAAGCCGTAAATTTTGGCATAAT

ATATGGCATAAGCGATTAT

GGGCTTTCTCGAGACCTTAAAATATCAAGAAAAGAAGCAAAAGAGTACATAAATAATTATT

TTGAAAGATATAAAGGAGT

AAAAGATTATATTGAAAAAATAGTACGATTTGCAAAAGAAAATGGCTATGTGACTACAATA

ATGAACAGAAGGAGATATA

<div align="center">

Fig. 1C

SUBSTITUTE SHEET (RULE 26)

</div>

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TTCCTGAAATAAACTCAAGAAATTTTACTCAAAGATCGCAGGCCGAAAGGTTAGCAATGAA

TGCTCCGATACAGGGAAGT

GCGGCTGATATAATAAAAATGGCAATGGTTAAGGTATACAACGATTTAAAAAAATTAAAGC

TTAAGTCTAAGCTTATATT

GCAAGTTCATGACGAGCTTGTAGTGGATACTTATAAGGATGAAGTAGATATCATAAAAAAG

ATACTTAAAGAAAATATGG

AAAATGTAGTGCAATTAAAAGTTCCTCTGGTTGTTGAAATTGGCGTAGGGCCTAATTGGTT

TTTGGCCAAGTGAGGTGTG

CAAAAATGCAAGTAATTGGACTTACTGGCGGCATTGCCTCAGGTAAAAGTACTGTATCCAA

GCTATTAAAAAAGATGGGA

GCTGTGGTAATTGATGCAGATATTGTATCAAGAGAAATAATGGTAAAAGGAACAGAAGCAT

ATAATAGAATTGTAGAATA

TTTTGGAAAAGAAATTTTAAAGGAAGACGGCGAAATTGACAGAAAGAAATTAGGCAATATT

GTGTTTGCTGACAGAAAAA

AGCTTAAAAAAACTAAATGAAATTACTCATCCCATAATAATAGAGAGGATAAAAGAAAAAAT

AGAAGAAGAAAGAAAAAAA

ATCAGCAAAAGGCAATTGTATTAGATGCAGCTCTTTTAATTGAGATGAACTTTATAAATGG

TAGATGAGGTTTGGTTAGT

AGTAGTAGACTCAAAAACTCAAATAAAGAGGGTTATGGAGAGGGATAAGCTTTCTTATAAG

GATGCGATAAACCGAATAA

AAAGCCAAATGCTTTGGACGAAAAAATGAAGTACGCAGATTTTATAATAAACATAGCAAAG

TTTTAAAGCTATGGAAAAA

CAGTTACACTATTTTGGGAAAGATTTGCAACGTAAATTCATGATATCGGAGGAGTTACTTT

GAGAAAAAAAGTTGCGGCA

ATTTTAATACTATTAAGTTTATTGTTTACTTACGAGTTAAATACTCATTATTTTTTAAAAA

AAATTTATCCTCGTAAATA

CTATAATTATGTATTTTATTACGCAAAAGAGTATGGCATGGACCCCTATCTTATTTTTGCT

GTGATAAAAGTCGAAAGTA

ATTTTAGAAGCGATGCAATTTCCAGCAAAAATGCTAGAGGGTTAATGCAAATT

Fig. 1D

TTACCAGAAACTGGAGAATGGATTGCT

AAAGAAATAGGGATAAAAAATTACAGCAATAGTATGCTTTTTGAACCTAAATATAATATTC

AAATGGGAACGTGGTATTT

GACTTATCTTCTTAAAACTTTTAATGGAAATATTAAATTGGCTCTAGCGGCCTATAACGGA

GGCAGTGGAAATGTAGATG

CATGGCTTAAAGATAAAAGATTTTCTAAAGATGGTAAACAACTACATATTGTTCCTTTTCC

TGAGACAAATAAATATATA

AAAAAGGTATTAGCAGTTTACGAGATCTATAAGTTCATATATGAAACTAAGAATTGATATG

AAGATTACTATATACAAAA

AAAGTTGAAAAATTGGTATAATAGTATCGTTAAAAAGAAAATAAAAAGGGAGGACACAAAT

GAAACCGTTGAAACATTTA

AAAGACTTAAAGGAAGTAAAAATCGAAAGAGATAGGGAGTTTTTCTCAGCCACTCATGAAG

AAATAAAAAATGCTTGGAC

TACAGATGTGTATTTTTTAAGAACCCAAGATATTTTATCATATCTTGGTGTACAGGATAAA

ATAGTTACTGCAGAGATAT

TTCCAAGAAAAAAAGGAGTTTTTGCAGGATTACCAGAAGTAATGAGTTTACTTAAAGACAA

AAATGTGGAAGTATGGTCT

TTGAAAGAAGGAGATACTTTTGAAGCTAAAGATACGGTAATGAGAATAAAAGGACCTTATA

GTGAATTTGGAATTTATGA

AACGGCAATATTAGGAATTC

Fig. 1E

```
1/1                                                    31/11
ATG TAT AAA TTT TTA ATA ATT GAT GGA AGT AGC CTC ATG TAC AGA GCC TAT TAT GCC TTG
 M   Y   K   F   L   I   I   D   G   S   S   L   M   Y   R   A   Y   Y   A   L

61/21                                                  91/31
CCC ATG CTT ACT ACA AGT GAG GGA TTG CCT ACA AAT GCT CTG TAT GGT TTT ACT ATG ATG
 P   M   L   T   T   S   E   G   L   P   T   N   A   L   Y   G   F   T   M   M

121/41                                                 151/51
CTT ATA AAA CTT ATC GAG GAG GAA AAA CCT GAT TAC ATA GCT ATT GCT TTT GAC AAA AAA
 L   I   K   L   I   E   E   E   K   P   D   Y   I   A   I   A   F   D   K   K

181/61                                                 211/71
GCT CCT ACT TTT AGA CAC AAA GAA TAT CAA GAC TAC AAA GCT ACA AGA CAA GCT ATG CCT
 A   P   T   F   R   H   K   E   Y   Q   D   Y   K   A   T   R   Q   A   M   P

241/81                                                 271/91
GAA GAA CTT GCT GAA CAA GTA GAC TAT TTG AAA GAA ATT ATA GAT GGC TTT AAT ATA AAG
 E   E   L   A   E   Q   V   D   Y   L   K   E   I   I   D   G   F   N   I   K

301/101                                                331/111
ACA TTA GAA TTA GAA GGT TAT GAA GCT GAT GAC ATT ATA GGG ACT ATT TCA AAG CTG GCA
 T   L   E   L   E   G   Y   E   A   D   D   I   I   G   T   I   S   K   L   A

361/121                                                391/131
GAG GAA AAA GGA ATG GAA GTG CTT GTA GTT ACA GGA GAC AGA GAT GCT CTT CAA TTA GTT
 E   E   K   G   M   E   V   L   V   V   T   G   D   R   D   A   L   Q   L   V

421/141                                                451/151
TCA GAT AAA GTG AAG ATA AAA ATT TCT AAA AAG GGT ATT ACT CAG ATG GAA GAG TTT GAC
 S   D   K   V   K   I   K   I   S   K   K   G   I   T   Q   M   E   E   F   D

481/161                                                511/171
GAA AAG GCT ATT TTA GAA AGG TAT GGA ATA ACT CCT CAG CAG TTT ATA GAT TTA AAA GGG
 E   K   A   I   L   E   R   Y   G   I   T   P   Q   Q   F   I   D   L   K   G

541/181                                                571/191
CTT ATG GGA GAT AAA TCT GAT AAT ATC CCT GGA GTA CCT AAT ATA GGG GAA AAA ACT GCG
 L   M   G   D   K   S   D   N   I   P   G   V   P   N   I   G   E   K   T   A

601/201                                                631/211
ATT AAG CTA TTA AAG GAT TTT GGA ACA ATT GAA AAT TTA ATC CAA AAT CTT TCT CAG CTT
 I   K   L   L   K   D   F   G   T   I   E   N   L   I   Q   N   L   S   Q   L

661/221                                                691/231
AAA GGT AAA ATA AAA GAA AAT ATA GAA AAC AAT AAA GAG TTA GCT ATA ATG AGT AAG AGG
```

Fig. 2A

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K    G    K    I    K    E    N    I    E    N         N    K    E    L    A    I    M    S    K    R
721/241                                      751/251
CTT GCT ACT ATA AAA AGA GAC ATT CCC ATT GAG ATA GAT TTT GAG GAG TAT AAA GTA AAA
L    A    T    I    K    R    D    I    P    I    E    I    D    F    E    E    Y    K    V    K
781/261                                      811/271
AAA TTT AAT GAG GAG AAG CTT TTA GAG CTT TTT AAT AAA TTA GAA TTC TTT AGT TTA ATT
K    F    N    E    E    K    L    L    E    L    F    N    K    L    E    F    F    S    L    I
841/281                                      871/291
GAT AAC ATA AAG AAA GAA AGT AGC ATA GAG ATT GTA GAT AAT CAT AAA GTT GAA AAA TGG
D    N    I    K    K    E    S    S    I    E    I    V    D    N    H    K    V    E    K    W
901/301                                      931/311
TCA AAA GTA GAT ATA AAA GAA TTA GTA ACT TTG TTG CAA GAT AAC AGA AAT ATT GCT TTT
S    K    V    D    I    K    E    L    V    T    L    L    Q    D    N    R    N    I    A    F
961/321                                      991/331
TAC CCG TTA ATT TAT GAA GGG GAA ATA AAA AAA ATA GCC TTT TCT TTT GGA AAG GAT ACG
Y    P    L    I    Y    E    G    E    I    K    K    I    A    F    S    F    G    K    D    T
1021/341                                     1051/351
GTT TAT ATT GAC GTT TTC CAA ACA GAA GAT TTA AAG GAG ATT TTT GAA AAA GAA GAT TTT
V    Y    I    D    V    F    Q    T    E    D    L    K    E    I    F    E    K    E    D    F
1081/361                                     1111/371
GAA TTT ACA ACC CAT GAA ATA AAG GAT TTT TTA GTG AGG CTT TCT TAT AAA GGA ATA GAG
E    F    T    T    H    E    I    K    D    F    L    V    R    L    S    Y    K    G    I    E
1141/381                                     1171/391
TGT AAA AGC AAG TAC ATA GAT ACT GCT GTA ATG GCT TAT CTT CTG AAT CCT TCT GAG TCT
C    K    S    K    Y    I    D    T    A    V    M    A    Y    L    L    N    P    S    E    S
1201/401                                     1231/411
AAC TAT GAC TTA GAC CGT GTG CTA AAA AAA TAT TTA AAG GTA GAT GTG CCT TCT TAT GAA
N    Y    D    L    D    R    V    L    K    K    Y    L    K    V    D    V    P    S    Y    E
1261/421                                     1291/431
GGA ATA TTT GGC AAA GGT AGG GAT AAA AAG AAA ATT GAA GAG ATT GAC GAA AAC ATA CTT
G    I    F    G    K    G    R    D    K    K    K    I    E    E    I    D    E    N    I    L
1321/441                                     1351/451
GCT GAT TAT ATT TGC AGT AGA TGT GTG TAT CTA TTT GAT TTA AAA GAA AAG CTG ATG AAT
A    D    Y    I    C    S    R    C    V    Y    L    F    D    L    K    E    K    L    M    N
1381/461                                     1411/471
TTT ATT GAA GAG ATG GAT ATG AAA AAA CTT CTA TTA GAA ATA GAA ATG CCT CTT GTA GAA
F    I    E    E    M    D    M    K    K    L    L    L    E    I    E    M    P    L    V    E
```

Fig. 2B

1441/481   1471/491
GTT TTA AAA TCA ATG GAG GTA AGT GGT TTT ACA TTG GAT AAA GAA GTT CTA AAA GAG CTT
 V   L   K   S   M   E   V   S   G   F   T   L   D   K   E   V   L   K   E   L
1501/501   1531/511
TCA CAA AAG ATA GAT GAT AGA ATA GGA GAA ATA CTA GAT AAA ATT TAT AAA GAG GCA GGA
 S   Q   K   I   D   D   R   I   G   E   I   L   D   K   I   Y   K   E   A   G
1561/521   1591/531
TAT CAA TTT AAT GTA AAT TCA CCT AAG CAA TTA AGT GAA TTT TTG TTT GAA AAG TTA AAC
 Y   Q   F   N   V   N   S   P   K   Q   L   S   E   F   L   F   E   K   L   N
1621/541   1651/551
TTA CCA GTA ATA AAG AAA ACA AAA ACA GGA TAC TCT ACG GAT TCT GAA GTT TTG GAA CAA
 L   P   V   I   K   K   T   K   T   G   Y   S   T   D   S   E   V   L   E   Q
1681/561   1711/571
TTG GTT CCT TAT AAT GAT ATT GTC AGC GAT ATA ATA GAG TAT CGG CAA CTT ACA AAA CTT
 L   V   P   Y   N   D   I   V   S   D   I   I   E   Y   R   Q   L   T   K   L
1741/581   1771/591
AAA TCT ACT TAT ATA GAT GGA TTT TTG CCT CTT ATG GAT GAA AAC AAT AGA GTA CAT TCT
 K   S   T   Y   I   D   G   F   L   P   L   M   D   E   N   N   R   V   H   S
1801/601   1831/611
AAT TTT AAA CAA ATG GTT ACT GCT ACA GGT AGA ATA AGC AGC ACC GAG CCA AAT CTA CAA
 N   F   K   Q   M   V   T   A   T   G   R   I   S   S   T   E   P   N   L   Q
1861/621   1891/631
AAT ATA CCT ATA AGA GAA GAG TTT GGC AGA CAA ATT AGA AGG GCT TTT ATT CCG AGG AGT
 N   I   P   I   R   E   E   F   G   R   Q   I   R   R   A   F   I   P   R   S
1921/641   1951/651
AGA GAT GGA TAT ATT GTT TCA GCA GAT TAT TCT CAG ATT GAA CTG AGG GTT TTA GCA CAT
 R   D   G   Y   I   V   S   A   D   Y   S   Q   I   E   L   R   V   L   A   H
1981/661   2011/671
GTT TCG GGA GAT GAA AAG CTA ATA GAA TCT TTT ATG AAT AAT GAA GAT ATA CAT TTA AGG
 V   S   G   D   E   K   L   I   E   S   F   M   N   N   E   D   I   H   L   R
2041/681   2071/691
ACA GCT TCG GAG GTT TTT AAA GTT CCT ATG GAA AAA GTT ACA CCG GAG ATG AGA AGA GCA
 T   A   S   E   V   F   K   V   P   M   E   K   V   T   P   E   M   R   R   A
2101/701   2131/711
GCA AAA GCC GTA AAT TTT GGC ATA ATA TAT GGC ATA AGC GAT TAT GGG CTT TCT CGA GAC
 A   K   A   V   N   F   G   I   I   Y   G   I   S   D   Y   G   L   S   R   D
2161/721   2191/731

Fig. 2C

SUBSTITUTE SHEET (RULE 26)

CTT AAA ATA TCA AGA AAA GAA GCA AAA GAG TAC ATA AAT AAT TAT TTT GAA AGA TAT AAA

L   K   I   S   R   K   E   A   K   E   Y   I   N   N   Y   F   E   R   Y   K

2221/741   2251/751

GGA GTA AAA GAT TAT ATT GAA AAA ATA GTA CGA TTT GCA AAA GAA AAT GGC TAT GTG ACT

G   V   K   D   Y   I   E   K   I   V   R   F   A   K   E   N   G   Y   V   T

2281/761   2311/771

ACA ATA ATG AAC AGA AGG AGA TAT ATT CCT GAA ATA AAC TCA AGA AAT TTT ACT CAA AGA

T   I   M   N   R   R   R   Y   I   P   E   I   N   S   R   N   F   T   Q   R

2341/781   2371/791

TCG CAG GCC GAA AGG TTA GCA ATG AAT GCT CCG ATA CAG GGA AGT GCG GCT GAT ATA ATA

S   Q   A   E   R   L   A   M   N   A   P   I   Q   G   S   A   A   D   I   I

2401/801   2431/811

AAA ATG GCA ATG GTT AAG GTA TAC AAC GAT TTA AAA AAA TTA AAG CTT AAG TCT AAG CTT

K   M   A   M   V   K   V   Y   N   D   L   K   K   L   K   L   K   S   K   L

2461/821   2491/831

ATA TTG CAA GTT CAT GAC GAG CTT GTA GTG GAT ACT TAT AAG GAT GAA GTA GAT ATC ATA

I   L   Q   V   H   D   E   L   V   V   D   T   Y   K   D   E   V   D   I   I

2521/841   2551/851

AAA AAG ATA CTT AAA GAA AAT ATG GAA AAT GTA GTG CAA TTA AAA GTT CCT CTG GTT GTT

K   K   I   L   K   E   N   M   E   N   V   V   Q   L   K   V   P   L   V   V

2581/861   2611/871

GAA ATT GGC GTA GGG CCT AAT TGG TTT TTG GCC AAG TGA

E   I   G   V   G   P   N   W   F   L   A   K   *

Fig. 2D

MKLLELFNKLEFFSLIDNIKKESSIEIVDNHKVEKWSKVDIKELVTLLQDNRNIAFYPLIYEG
EIK
KIAFSFGKDTVYIDVFQTEDLKEIFEKEDFEFTTHEIKDFLVRLSYKGIECKSKYIDT
AVMAYLL
NPSESNYDLDRVLKKYLKVDVPSYEGIFGKGRDKKKIEEIDENILADYICSRCVYLFDLKEK
L
MNFIEEMDMKKLLLEIEMPLVEVLKSMEVSGFTLDKEVLKELSQKIDDRIGEILDKIYKEA
GY
QFNVNSPKQLSEFLFEKLNLPVIKKTKTGYSTDSEVLEQLVPYNDIVSDIIEYRQLTKLKSTY
ID
GFLPLMDENNRVHSNFKQMVTATGRISSSEANLQNIPIREEFGRQIRRAFIPRSRDGYIVSAD
Y
SQIELRVLAHVSGDEKLIESFMNNEDIHLRTASEVFKVPMEKVTPEMRRAAKAVNFGIIYGI
SD
YGLSRDLKISRKEAKEYINNYFERYKGVKDYIEKIVRFAKENGYVTTIMNRRRYIPEINSRN
FT
QRSQAERLAMNAPIQGSAADIIKMAMVKVYNDLKKLKLKSKLILQVHDELVVDTYKDEV
DII
KKILKENMENVVQLKVPLVVEIGVGPNWFLAK*

Fig. 3

```
1/1                                          31/11
ATG AAA GTT GAA AAA TGG TCT AAA GTA GAC ATC AAA GAA TTA GTA ACT TTG TTG CAA GAT
 M   K   V   E   K   W   S   K   V   D   I   K   E   L   V   T   L   L   Q   D
61/21                                        91/31
AAC AGA AAT ATT GCT TTT TAC CCG TTA ATT TAT GAA GGG GAA ATA AAA AAA ATA GCC TTT
 N   R   N   I   A   F   Y   P   L   I   Y   E   G   E   I   K   K   I   A   F
121/41                                       151/51
TCT TTT GGA AAG GAT ACG GTT TAT ATT GAC GTT TTC CAA ACA GAA GAT TTA AAG GAG ATT
 S   F   G   K   D   T   V   Y   I   D   V   F   Q   T   E   D   L   K   E   I
181/61                                       211/71
TTT GAA AAA GAA GAT TTT GAA TTT ACA ACC CAT GAA ATA AAG GAT TTT TTA GTG AGG CTT
 F   E   K   E   D   F   E   F   T   T   H   E   I   K   D   F   L   V   R   L
241/81                                       271/91
TCT TAT AAA GGA ATA GAG TGT AAA AGC AAG TAC ATA GAT ACT GCT GTA ATG GCT TAT CTT
 S   Y   K   G   I   E   C   K   S   K   Y   I   D   T   A   V   M   A   Y   L
301/101                                      331/111
CTG AAT CCT TCT GAG TCT AAC TAT GAC TTA GAC CGT GTG CTA AAA AAA TAT TTA AAG GTA
 L   N   P   S   E   S   N   Y   D   L   D   R   V   L   K   K   Y   L   K   V
361/121                                      391/131
GAT GTG CCT TCT TAT GAA GGA ATA TTT GGC AAA GGT AGG GAT AAA AAG AAA ATT GAA GAG
 D   V   P   S   Y   E   G   I   F   G   K   G   R   D   K   K   K   I   E   E
421/141                                      451/151
ATT GAC GAA AAC ATA CTT GCT GAT TAT ATT TGC AGT AGA TGT GTG TAT CTA TTT GAT TTA
 I   D   E   N   I   L   A   D   Y   I   C   S   R   C   V   Y   L   F   D   L
481/161                                      511/171
AAA GAA AAG CTG ATG AAT TTT ATT GAA GAG ATG GAT ATG AAA AAA CTT CTA TTA GAA ATA
 K   E   K   L   M   N   F   I   E   E   M   D   M   K   K   L   L   L   E   I
541/181                                      571/191
GAA ATG CCT CTT GTA GAA GTT TTA AAA TCA ATG GAG GTA AGT GGT TTT ACA TTG GAT AAA
 E   M   P   L   V   E   V   L   K   S   M   E   V   S   G   F   T   L   D   K
601/201                                      631/211
GAA GTT CTA AAA GAG CTT TCA CAA AAG ATA GAT GAT AGA ATA GGA GAA ATA CTA GAT AAA
 E   V   L   K   E   L   S   Q   K   I   D   D   R   I   G   E   I   L   D   K
661/221                                      691/231
ATT TAT AAA GAG GCA GGA TAT CAA TTT AAT GTA AAT TCA CCT AAG CAA TTA AGT GAA TTT
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Fig. 4A

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    I   Y   K   E   A   G   Y   Q   F   N   V   N   S   P   K   Q   L   S   E   F
721/241                                         751/251
TTG TTT GAA AAG TTA AAC TTA CCA GTA ATA AAG AAA ACA AAA ACA GGA TAC TCT ACG GAT
    L   F   E   K   L   N   L   P   V   I   K   K   T   K   T   G   Y   S   T   D
781/261                                         811/271
TCT GAA GTT TTG GAA CAA TTG GTT CCT TAT AAT GAT ATT GTC AGC GAT ATA ATA GAG TAT
    S   E   V   L   E   Q   L   V   P   Y   N   D   I   V   S   D   I   I   E   Y
841/281                                         871/291
CGG CAA CTT ACA AAA CTT AAA TCT ACT TAT ATA GAT GGA TTT TTG CCT CTT ATG GAT GAA
    R   Q   L   T   K   L   K   S   T   Y   I   D   G   F   L   P   L   M   D   E
901/301                                         931/311
AAC AAT AGA GTA CAT TCT AAT TTT AAA CAA ATG GTT ACT GCT ACA GGT AGA ATA AGC AGC
    N   N   R   V   H   S   N   F   K   Q   M   V   T   A   T   G   R   I   S   S
961/321                                         991/331
AGC GAG GCA AAT CTA CAA AAT ATA CCT ATA AGA GAA GAG TTT GGC AGA CAA ATT AGA AGG
    S   E   A   N   L   Q   N   I   P   I   R   E   E   F   G   R   Q   I   R   R
1021/341                                        1051/351
GCT TTT ATT CCG AGG AGT AGA GAT GGA TAT ATT GTT TCA GCA GAT TAT TCT CAG ATT GAA
    A   F   I   P   R   S   R   D   G   Y   I   V   S   A   D   Y   S   Q   I   E
1081/361                                        1111/371
CTG AGG GTT TTA GCA CAT GTT TCG GGA GAT GAA AAG CTA ATA GAA TCT TTT ATG AAT AAT
    L   R   V   L   A   H   V   S   G   D   E   K   L   I   E   S   F   M   N   N
1141/381                                        1171/391
GAA GAT ATA CAT TTA AGG ACA GCT TCG GAG GTT TTT AAA GTT CCT ATG GAA AAA GTT ACA
    E   D   I   H   L   R   T   A   S   E   V   F   K   V   P   M   E   K   V   T
1201/401                                        1231/411
CCG GAG ATG AGA AGA GCA GCA AAA GCC GTA AAT TAT GGC ATA ATA TAT GGC ATA AGC GAT
    P   E   M   R   R   A   A   K   A   V   N   Y   G   I   I   Y   G   I   S   D
1261/421                                        1291/431
TAT GGG CTT TCT CGA GAC CTT AAA ATA TCA AGA AAA GAA GCA AAA GAG TAC ATA AAT AAT
    Y   G   L   S   R   D   L   K   I   S   R   K   E   A   K   E   Y   I   N   N
1321/441                                        1351/451
TAT TTT GAA AGA TAT AAA GGA GTA AAA GAT TAT ATT GAA AAA ATA GTA CGA TTT GCA AAA
    Y   F   E   R   Y   K   G   V   K   D   Y   I   E   K   I   V   R   F   A   K
1381/461                                        1411/471
GAA AAT GGC TAT GTG ACT ACA ATA ATG AAC AGA AGG AGA TAT ATT CCT GAA ATA AAC TCA
    E   N   G   Y   V   T   T   I   M   N   R   R   R   Y   I   P   E   I   N   S
1441/481                                        1471/491
```
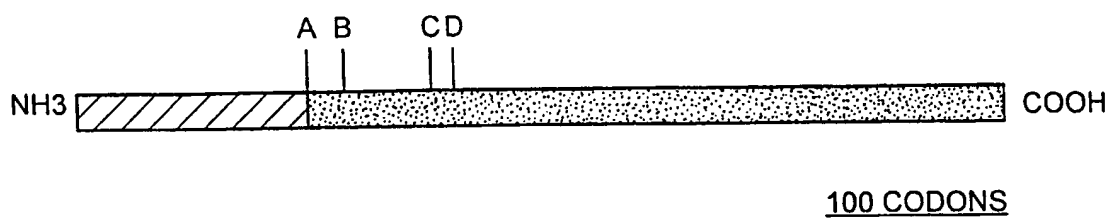
Fig. 4B

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AGA AAT TTT ACT CAA AGA TCG CAG GCC GAA AGG TTA GCA ATG AAT GCT CCG ATA CAG GGA
 R   N   F   T   Q   R   S   Q   A   E   R   L   A   M   N   A   P   I   Q   G
1501/501                                  1531/511
AGT GCG GCT GAT ATA ATA AAA ATG GCA ATG GTT AAG GTA TAC AAC GAT TTA AAA AAA TTA
 S   A   A   D   I   I   K   M   A   M   V   K   V   Y   N   D   L   K   K   L
1561/521                                  1591/531
AAG CTT AAG TCT AAG CTT ATA TTG CAA GTT CAT GAC GAG CTT GTA GTG GAT ACT TAT AAG
 K   L   K   S   K   L   I   L   Q   V   H   D   E   L   V   V   D   T   Y   K
1621/541                                  1651/551
GAT GAA GTA GAT ATC ATA AAA AAG ATA CTT AAA GAA AAT ATG GAA AAT GTA GTG CAA TTA
 D   E   V   D   I   I   K   K   I   L   K   E   N   M   E   N   V   V   Q   L
1681/561                                  1711/571
AAA GTT CCT CTG GTT GTT GAA ATT GGC GTA GGG CCT AAT TGG TTT TTG GCC AAG TGA
 K   V   P   L   V   V   E   I   G   V   G   P   N   W   F   L   A   K   *
```
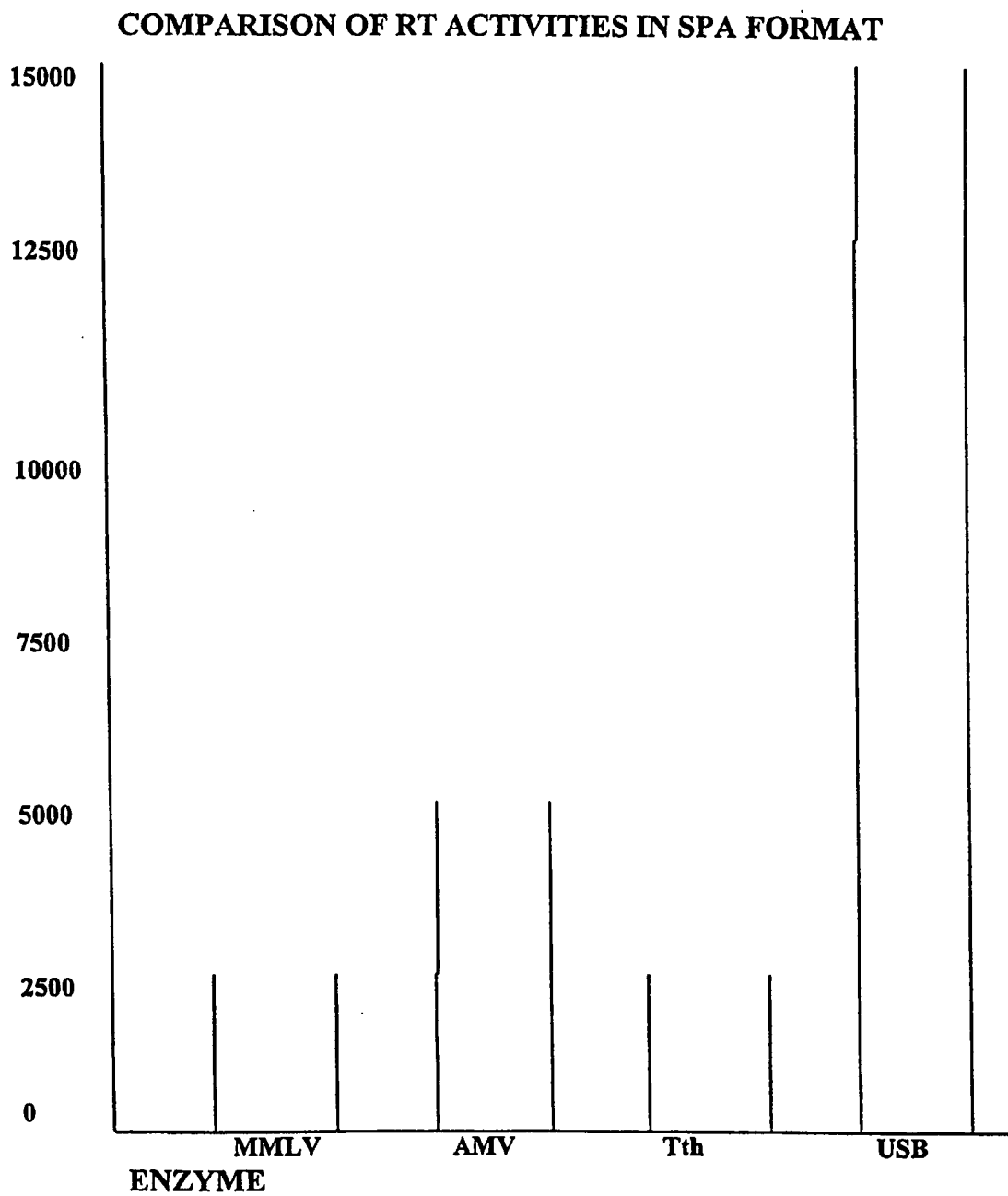
Fig. 4C

Fig. 5

COMPARISON OF RT ACTIVITIES IN SPA FORMAT

Fig. 6

ATGGCTTATAAATTTTTAATCATTGATGGTAGTAGCCTCATGTACAGAG

CCTATTATGCCTTGCCCATGCTTACTACAAGTGAGGGATTGCCTACAAATGCTCTGTA

TGGTTTTACTATGATGCTTATA

AAACTTATCGAGGAGGAAAAACCTGATTACATAGCTATTGCTTTTGACAAAAAAGCT

CCTACTTTTAGACACAAAGAATA

TCAAGACTACAAAGCTACAAGACAAGCTATGCCTGAAGAACTTGCTGAACAAGTAGA

CTATTTGAAAGAAATTATAGATG

GCTTTAATATAAAGACATTAGAATTAGAAGGTTATGAAGCTGATGACATTATAGGGA

CTATTTCAAAGCTGGCAGAGGAA.

AAAGGAATGGAAGTGCTTGTAGTTACAGGAGACAGAGATGCTCTTCAATTAGTTTCA

GATAAAGTGAAGATAAAAATTTC

TAAAAAGGGTATTACTCAGATGGAAGAGTTTGACGAAAGGCTATTTTAGAAAGGTA

TGGAATAACTCCTCAGCAGTTTA

TAGATTTAAAAGGGCTTATGGGAGATAAATCTGATAATATCCCTGGAGTACCTAATAT

AGGGGAAAAAACTGCGATTAAG

CTATTAAAGGATTTTGGAACAATTGAAAATTTAATCCAAAATCTTTCTCAGCTTAAAG

GTAAAATAAAAGAAAATATAGA

AAACAATAAAGAGTTAGCTATAATGAGTAAGAGGCTTGCTACTATAAAAAGAGACAT

TCCCATTGAGATAGATTTTGAGG

AGTATAAAGTAAAAAAATTTAATGAGGAGAAGCTTTTAGAGCTTTTTAATAAATTAGA

ATTCTTTAGTTTAATTGATAAC

ATAAAGAAAGAAAGTAGCATAGAGATTGTAGATAATCATAAAGTTGAAAAATGGTCA

AAAGTAGATATAAAAGAATTAGT

AACTTTGTTGCAAGATAACAGAAATATTGCTTTTTACCCGTTAATTTATGAAGGGGAA

ATAAAAAAAAATAGCCTTTTCTT

Fig. 7A

17/18

TTGGAAAGGATACGGTTTATATTGACGTTTTCCAAACAGAAGATTTAAAGGAGATTTT
TGAAAAAGAAGATTTTGAATTT
ACAACCCATGAAATAAAGGATTTTTTAGTGAGGCTTTCTTATAAAGGAATAGAGTGTA
AAAGCAAGTACATAGATACTGC
TGTAATGGCTTATCTTCTGAATCCTTCTGAGTCTAACTATGACTTAGACCGTGTGCTAA
AAAAATATTTAAAGGTAGATG
TGCCTTCTTATGAAGGAATATTTGGCAAAGGTAGGGATAAAAAGAAAATTGAAGAGA
TTGACGAAAACATACTTGCTGAT
TATATTTGCAGTAGATGTGTGTATCTATTTGATTTAAAAGAAAAGCTGATGAATTTTAT
TGAAGAGATGGATATGAAAAA
ACTTCTATTAGAAATAGAAATGCCTCTTGTAGAAGTTTTAAAATCAATGGAGGTAAGT
GGTTTTACATTGGATAAAGAAG
TTCTAAAAGAGCTTTCACAAAAGATAGATGATAGAATAGGAGAAATACTAGATAAAA
TTTATAAAGAGGCAGGATATCAA
TTTAATGTAAATTCACCTAAGCAATTAAGTGAATTTTTGTTTGAAAAGTTAAACTTACC
AGTAATAAAGAAAACAAAAAC
AGGATACTCTACGGATTCTGAAGTTTTGGAACAATTGGTTCCTTATAATGATATTGTC
AGCGATATAATAGAGTATCGGC
AACTTACAAAACTTAAATCTACTTATATAGATGGATTTTTGCCTCTTATGGATGAAAA
CAATAGAGTACATTCTAATTTT
AAACAAATGGTTACTGCTACAGGTAGAATAAGCAGCACCGAGCCAAATCTACAAAAT
ATACCTATAAGAGAAGAGTTTGG
CAGACAAATTAGAAGGGCTTTTATTCCGAGGAGTAGAGATGGATATATTGTTTCAGCA
GATTATTCTCAGATTGAACTGA
GGGTTTTAGCACATGTTTCGGGAGATGAAAAGCTAATAGAATCTTTTATGAATAATGA

Fig. 7B

AGATATACATTTAAGGACAGCT

TCGGAGGTTTTTAAAGTTCCTATGGAAAAAGTTACACCGGAGATGAGAAGAGCAGCA

AAAGCCGTAAATTTTGGCATAAT

ATATGGCATAAGCGATTATGGGCTTTCTCGAGACCTTAAAATATCAAGAAAAGAAGC

AAAAGAGTACATAAATAATTATT

TTGAAAGATATAAAGGAGTAAAAGATTATATTGAAAAAATAGTACGATTTGCAAAAG

AAAATGGCTATGTGACTACAATA

ATGAACAGAAGGAGATATATTCCTGAAATAAACTCAAGAAATTTTACTCAAAGATCG

CAGGCCGAAAGGTTAGCAATGAA

TGCTCCGATACAGGGAAGTGCGGCTGATATAATAAAAATGGCAATGGTTAAGGTATA

CAACGATTTAAAAAAATTAAAGC

TTAAGTCTAAGCTTATATTGCAAGTTCATGACGAGCTTGTAGTGGATACTTATAAGGA

TGAAGTAGATATCATAAAAAAG

ATACTTAAAGAAAATATGGAAAATGTAGTGCAATTAAAAGTTCCTCTGGTTGTTGAAA

TTGGCGTAGGGCCTAATTGGTT

TTTGGCCAAGTGA

Fig. 7C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/05612

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C07H 21/04; C12N 1/14, 1/20, 5/00, 9/12, 15/00; C12P 19/34
US CL   :435/91.1, 91.2, 91.51, 194, 252.3, 254.11, 320.1, 325; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/91.1, 91.2, 91.51, 194, 252.3, 254.11, 320.1, 325; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, CAPLUS
thermoanaerobacter, thermohydrosulfuricus, DNA polymerase,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 21821 A1(AMERSHAM LIFE SCIENCE, INC.)19 June 1997 (see whole document)	1-651
P, A	US 5,744,312 A (MAMONE et al.) 28 April 1998 (see whole document)	1-651

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
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"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 JUNE 1999	1 2 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer EINAR STOLE
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